ANTIBACTERIAL ACTIVITIES OF SELECTED MARINE FUNGI FROM PENINSULAR MALAYSIA

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ABSTRACT

Over the past few decades, scientists have come to acknowledge the growing importance of marine and marine-related resources for new drugs, pesticides and anti-fouling substances, with over 10,000 metabolites isolated from marine organisms to date. In an effort to tap into the pharmaceutical potential of marine fungi in Malaysia, selected marine derived endophytic and manglicolous fungi from the coastlines of Peninsular Malaysia were investigated for their antibacterial potential. The endophytic fungi in this study were isolated from two marine associated plants; *Vitex rotundifolia* L.f. and *Ipomoea pes-caprae* (L.) R.Br., from the east and west coast of Peninsular Malaysia respectively. A total of 13 and 21 endophytic fungi were isolated from *V. rotundifolia* and *I. pes-caprae*, respectively. The eleven manglicolous fungi strains used in this study were of mangrove origin and were obtained from the Institute of Biological Sciences (ISB) culture collection. In preliminary experiments, a mycelial plug assay was employed to study the antibacterial activities of both endophytic and manglicolous fungi. Almost 38.5% of the fungal strains from *V. rotundifolia* and 47.6% of the fungal strains from *I. pes-caprae* exhibited antibacterial activity against at least one test bacteria. Ten or 91% of the manglicolous fungi exhibited antibacterial activity against at least one test bacteria. Endophytic fungi from *I. pes-caprae* displayed higher potential for antibacterial activity in comparison to endophytic fungi from *V. rotundifolia*. *Minimidochium* sp. and *Bipolaris* sp. displayed antibacterial activity towards five or more of the test bacteria. Ten or 91% of the eleven manglicolous fungi displayed antibacterial activity. *Saccardoella rhizophorae*, *Henningsomyces* sp., *Dactylospora haliotrepha* (ISB003) and MF 28 exhibited promising antibacterial potential. Based on the results from the mycelial plug assay, five endophytic and ten manglicolous fungi were further investigated using the broth microdilution assay to obtain quantitative antibacterial parameters. *Saccardoella rhizophorae* was chosen for complete chemical

analysis of its secondary metabolite profile. Bioactivity-guided fractionation of *S. rhizophorae* crude extract resulted in the isolation and characterization of two fatty acids namely palmitic acid and linoleic acid from the *n*-hexane fraction. Both isolated fatty acids exhibited antibacterial activity against gram positive bacteria. Certain fatty acids are important constituents of essential oils and fatty acids, such as linoleic acid, are important as nutritional supplements. Fatty acids are also essential in the field of biodiesel which is fast becoming the preferred alternate to conventional fuel with research being performed to identify sustainable sources of biodiesel feedstock. The isolation of fatty acids from *S. rhizophorae* indicates its potential application in the field of food technology and biodiesel. Although Malaysia hosts a great diversity of fungi, there are very few studies on metabolites of fungal origin, thus this study may prove crucial in paving the way towards commercial application of fungal metabolites and possibly lead to further discovery of new or novel fungal metabolites with pharmacological values.

ABSTRAK

Sejak beberapa dekad yang lalu, ahli sains telah mengakui kepentingan sumber marin di dalam kajian untuk ubat-ubatan baru, racun perosak dan bahan-bahan yang mempunyai potensi aktiviti biologi. Laporan kaji selidik santifik telah melaporkan lebih kurang 10,000 metabolit kimia yang telah diasingkan daripada organisma marin terutamanya, span, karang lembut, kulat dan bakteria di seluruh dunia. Dalam usaha untuk meneroka potensi farmaseutikal daripada kulat marin di Malaysia, kulat marin berasal kulat endofitik dan saprofitik dari pantai Semenanjung Malaysia telah dipilih untuk kajian selidik potensi antibakteria mereka. Kulat endofitik dalam kajian ini telah diasingkan daripada dua tumbuhan marin iaitu; *Vitex rotundifolia* L.f dan *Ipomoea pes-caprae* (L.) R.Br., dari pantai timur dan pantai barat Semenanjung Malaysia, masing-masing. Sebanyak 13 dan 21 kulat endofitik telah diasingkan daripada *V. rotundifolia* dan *I. pes-caprae* masing-masing. Sebelas kulat daripada paya bakau yang dikaji dalam kajian ini berasal daripada kayu bakau dan diperolehi daripada koleksi kultur Institut Sains Biologi. Dalam percubaan awal, “plug assay” telah digunakan untuk mengkaji aktiviti antibakteria kedua-dua jenis kulat. Hampir 38.5% daripada kulat endofitik daripada *V. rotundifolia* dan 47.6% daripada kulat endofitik daripada *I. pes-caprae* memaparkan aktiviti antibakteria terhadap sekurang-kurangnya satu bakteria ujian dalam penskrinan awal. Keputusan yang diperolehi menunjukkan bahawa kulat endofitik yang dipencil daripada tumbuhan *I. pes-caprae* memaparkan potensi aktiviti antibakteria yang lebih tinggi berbanding dengan kulat endofitik yang dipencil daripada tumbuhan *V. rotundifolia* terutama kulat seperti, *Minimidochium* sp. dan *Bipolaris* sp. yang memaparkan aktiviti antibakteria terhadap lima atau lebih daripada bakteria yang diuji. Daripada sebelas kulat yang diperolehi daripada paya bakau, sepuluh atau 91% daripada kulat tersebut memaparkan aktiviti antibakteria yang ketara. Kulat seperti, *Saccardoella rhizophorae*, *Henningsomyces* sp., *Dactylospora haliotrepha* (ISB003) dan MF 28 telah

memaparkan potensi antibakteria yang memberangsangkan. Berdasarkan keputusan antibakteria awal, lima kulat endofitik dan sembilan kulat daripada paya bakau telah disiasat secara lanjut melalui “broth microdilution assay” untuk mendapatkan parameter antibakteria yang lebih kuantitatif. *Saccardoella rhizophorae* sejenis kulat saprofitik telah dipilih untuk analisis kimia lengkap terhadap profil metabolit sekundernya. Pengasingan metabolit berpandu bioaktiviti ke atas ekstrak mentah *S. rhizophorae* menyebabkan pengasingan dan pencirian dua asid lemak iaitu asid palmitic dan asid linoleik daripada pecahan *n*-heksana. Kedua-dua asid lemak yang diasingkan mempamerkan aktiviti antibakteria terhadap bakteria gram positif. Asid lemak tertentu adalah juzuk penting dalam minyak yang penting dan asid lemak seperti asid linoleik penting sebagai pemakanan tambahan. Selain itu, asid lemak yang juga penting dalam bidang biodiesel yang kini menjadi alternatif pilihan kepada bahan api konvensional dengan penyelidikan yang berterusan sedang dilakukan untuk mengenal pasti sumber-sumber yang mampan bahan mentah biodiesel. Pengasingan asid lemak dari *S. rhizophorae* menunjukkan aplikasi yang berpotensi dalam bidang teknologi makanan dan biodiesel. Walaupun Malaysia menjadi tuan rumah kepada diversiti besar kulat, setakat ini hanya terdapat sedikit kajian mengenai metabolit daripada kulat. Oleh demikian, kajian ini membuktikan kepentingan halatuju ke arah aplikasi komersil metabolit kulat dan mungkin membawa kepada penemuan metabolit kulat baru atau novel dengan nilai farmakologi pada masa depan.

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Remember to look up at the stars and not down at your feet. Try to make sense of what you see and wonder about what makes the universe exist. Be curious. And however difficult life may seem there is always something you can do and succeed at. It matters that you don't just give up. – Stephen Hawking

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LIST OF SYMBOLS AND ABBREVIATIONS

ARDRA	Amplified rDNA restriction analysis
ARISA	Amplified ribosomal intergenic spacer analysis
ASTM	American society for testing and materials
ATCC	American type culture collection
B. cereus	*Bacillus cereus*
BLAST	Basic local alignment search tool
B. subtilis	*Bacillus subtilis*
$C_{16}H_{32}O_2$	Palmitic acid
$C_{18}H_{32}O_2$	Linoleic acid
CDC	Centers for Disease Control
$CDCl_3$	Chloroform D1
cfu	Colony forming unit
CMA	Corn meal agar
C. cladosporioides	*Clasdosporium cladosporioides*
C. eragrostidis	*Cochliobolus eragrostidis*
C. hippeastri	*Colletotrichum hippeastri*
C. lunatus	*Cochliobolus lunatus*
C. maritima	*Corollospora maritima*
Cl	Chloride
CLSI	Clinical and Laboratory Standards Institute
°C	Degree Celsius
COSY	Correlation spectroscopy
DEPT	Distortionless enhancement by polarization transfer
DGGE	Denaturing gradient gel electrophoresis
D. haliotrepha	*Dactylospora haliotrepha*
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DS	Dichloromethane fraction of *Saccardoella rhizophorae* crude
EA	Ethyl acetate
EAS	Ethyl acetate crude extract of *Saccardoella rhizophorae*
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
E. faecalis	*Enterococcus faecalis*
EIMS	Electron impact mass spectroscopy
ESBL	Extended Spectrum Beta Lactamase
EUCAST	European committee on antimicrobial susceptibility testing
FT-IR	Fourier transform infrared spectroscopy
F. equiseti	*Fusarium equiseti*
G. mangiferae	*Guignardia mangiferae*
HS	*n*-hexane fraction of *Saccardoella rhizophorae* crude
HMBC	Heteronuclear multiple-bond correlation spectroscopy
HSQC	Heteronuclear single-quantum correlation spectroscopy
INT	p-Iodonitrotetrazolium violet
I. pes-caprae	*Ipomoea pes-caprae*
IR	Infra-red
ISB	Institute of Biological Science
ITS	Inter-transcribed spacer
L. helminthicola	*Letendraea helminthicola*
LC-MS	Liquid chromatography-mass spectrometry
mg	milligram
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
ml	Millilitre

M. luteus	*Micrococcus luteus*
mm	Millimetre
mM	Milimolar
MS	Methanol fraction of *Saccardoella rhizophorae* crude
MTCC	Microbial type culture collection and Gene Bank
N. primolutea	*Nemania primolutea*
NCBI	National Centre for Biotechnology Information
NMR	Nuclear magnetic resonance
nm	Nanometre
NRPS	Nonribosomal peptide synthethase
LB	Luria broth
LPS	Lipo-polysaccharide
LTSs	Lowest taxonomic units
P. aeruginosa	*Pseudomonas aeruginosa*
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PCR	Polymerase chain reaction
PKS	Polyketide synthase
ppm	Parts per million
PUFA	Polyunsaturated fatty acids
rpm	Rotations per minute
rRNA	Ribosomal ribonucleic acid
S. aureus	*Staphylococcus aureus*
S. rhizophorae	*Saccardoella rhizophorae*
SSCP	Single-strand conformation polymorphism
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TGGE	Temperature gradient gel electrophoresis

TLC	Thin layer chromatography
T-RFLP	Terminal restriction fragment length polymorphism
TRIS	Tris(hydroxymethyl)aminomethane
µg	Microgram
µl	Microliter
US	United States
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
V. enalia	*Verruculina enalia*
V. minor	*Vinca minor*
V. rotundifolia	*Vitex rotundifolia*
WHO	World Health Organization
%	Percentage

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INTRODUCTION

The marine environment comprises 70% of the Earth's surface; however taking into account the topography of the ocean floor, the actual surface area of the ocean is considerably higher. The ocean harbours great biological diversity accounting for more than 90% of the whole biosphere (Subramani *et al*., 2012). This estimation could be in fact higher due underestimated oceanic depth which stands at slightly more than 10,000m in comparison to the vertical rise of land, the Mt. Everest at merely 8848m thus suggesting the existence of greater biodiversity in the oceans (Fautin, 2012; Manivasagan *et al*., 2013). However, most reports of natural product discovery stem from the terrestrial environment due to the underexplored state of the marine habitat (Dias *et al*., 2012).

Microorganisms such as bacteria and fungi, have been evolving for the past 3.8 billion years and are shown to produce more evolved metabolites as a part of their defence mechanism thus making them candidates for the production of bioactive natural products to be used as drug leads (Joel *et al*., 2013; Swathi *et al*., 2013). Scientists' hypothesize that the need of these organisms to adapt and survive in the considerably harsh marine environment may have shaped their biochemical pathways into producing unique secondary metabolites (Zhou *et al*., 2014).

The marine habitat is host to a wide variety of organisms particularly microorganisms that are promising sources for the discovery of novel metabolites (Manivasagan *et al*., 2013). Recent years has seen a growing interest in marine derived fungi as sources of new or novel metabolites (Zhou *et al*., 2014). Marine derived fungi produce bioactive metabolites with wide range of activities such as anticancer, antibacterial, antiplasmodial, antiviral and anti-inflammatory (Ebel, 2012; Duarte *et al*., 2012). Interest in marine derived fungi as producers of antibacterial metabolites

commenced with the discovery of cephalosporin C by the fungi *Cephalosporium acremonium* in 1940's (Proksch *et al*., 2008). Currently, marine derived fungi represent a large fraction of antibacterial compound producers in comparison to other microbial flora (Bhatnagar *et al*., 2012).

Two-thirds of drugs used in the treatment of human diseases over the past 25 years, have been derived from natural products or derivatives of them (Berkov *et al*., 2014). Technological and molecular biology advances have opened up new frontiers in natural product research, allowing effective investigation of new leads from Nature (Berkov *et al*., 2014). Hastened human practices and inappropriate use of antibiotics has led to an increase in bacterial resistance to clinically used antibiotics often leading to failure of conventional antibiotic therapies (Ola *et al*., 2014; Rao, *et al*., 2014). This scenario further raises the urgency to develop new antibiotics with novel mechanisms of action to counter effect the current resistance mechanisms of bacteria (Ola *et al*., 2014; Gomes *et al*., 2014).

There is a general lack of information and few studies on bioactive metabolites from marine derived fungi from Malaysia. A study by Zainuddin *et al*. (2010), investigated the antimicrobial properties of 152 marine derived fungi from Malaysia and successfully isolated 2,2,7-trimethyl-2H-chromen-5-ol from the marine derived fungi *Fasciatispora nypae* K.D. Hyde, a compound that was never before reported to be isolated from nature. The study by Zainuddin *et al*. (2010) only targeted the bioactivities of mangrove fungi; however there are other ecological groups of marine derived fungi such as algicolous, arenicolous, endophytic fungi from coastal associated plants and others that have yet to be explored in Malaysia.

Endophytic fungi are known to be excellent sources of a wide array of new biologically active metabolites (Debbab *et al*., 2011; Saleem *et al*., 2013) with a great number of compounds isolated in the past decades (Zhang *et al*., 2014). However there

have only been few reports from Malaysia (Sultan *et al*., 2011; Tong *et al*., 2011) on the potential of bioactive metabolites of endophytic fungi, all originating from medicinal herbs and non-marine associated plants of Malaysia. The marine associated plants, *Vitex rotundifolia* and *Ipomoea pes-caprae* are usually found as crawlers along the sand dunes, which is an area located in the intertidal are, that makes them both excellent sources for endophytic fungi. Mangroves are also located in the intertidal zone and are known to be host to a great diversity of fungi due to its high carbon recycling rates (Sridhar *et al*., 2012). Manglicolous fungi have been reported to produce a broad variety of new and novel bioactive metabolites with significant pharmacological values (Joel *et al*., 2013). Studies on bioactivities of manglicolous fungi from Malaysia are still at its initial stages while the bioactivities of fungi from the marine associated plants in the present study have not been previously explored in Malaysia. There have been no studies on the bioactivities of endophytic fungi from *V. rotundifolia,* while there was only one study (Guo *et al*., 2012) pertaining to the bioactivity of endophytic fungi from *I. pes-caprae* however this study was not reported from Malaysia. There have also been no reports from Malaysia regarding metabolites isolated from the manglicolous fungi in the present study. Marine derived fungi from Malaysia may harbour promising potential for the discovery of pharmacologically significant metabolites.

Research objectives:

1. To isolate and identify endophytic fungi from marine associated plants, *Vitex rotundifolia* and *Ipomoea pes-caprae* using Inter-trancribed spacer (ITS) sequence analysis.

2. To evaluate the antibacterial activity of endophytic fungi from marine associated plants (*V. rotundifolia* and *I. pes-caprae*) and manglicolous fungi from mangrove decaying materials of Peninsular Malaysia using mycelial plug and broth microdilution assay
3. To isolate and characterize the antibacterial secondary metabolites from the manglicolous fungi, *Saccardoella rhizophorae* by using bioactivity-guided fractionation coupled with Nuclear magnetic resonance (NMR) spectroscopy, Liquid chromatography-mass spectrometry (LC-MS), Ultra-violet (UV) spectroscopy and Infra-red (IR) spectroscopy.

CHAPTER 2

LITERATURE REVIEW

2.1 Marine organisms and their potential as producers of natural products

Nature is known to be a reservoir of compounds with high chemical diversity and excellent therapeutic potential (Bhatnagar *et al.*, 2012). Approximately 70% of the earth's surface constitutes water and every life form on earth is believed to have its origin in the sea (Haefner, 2003). Some marine biologists go as far as to believe that in certain marine ecosystems, the biodiversity can be even higher than what is usually observed in tropical rainforests (Haefner, 2003). The search for natural products was traditionally limited to plants especially higher terrestrial plants; however over the years, the scientific world has become increasingly aware of the vast untapped potential of natural products from marine life forms (Ebel, 2012).

The discovery of natural products from marine sources began in the 1950's, which resulted in the isolation of two nucleosides; spongouridine and spongothymidine from the sponge *Tectitethya crypta* Laubenfels (Oliveira *et al.*, 2012). This pioneering discovery led to the synthesis of the anticancer drug Cytarabine, used in the treatment of acute myeloid leukemia and the antiviral drug Vidarabine, used in the treatment of herpes virus infection (Oliveira *et al.*, 2012). Marine organisms live in an environment significantly different from its terrestrial counterparts, thereby suggesting that the secondary metabolite profiles of marine organism may differ in terms of its chemical structures and pharmacological activities (Lu *et al.*, 2010; Debbab *et al.*, 2013). The marine ecosystem is known to be governed by harsh biological, physical and chemical parameters which may have given rise to novel metabolic pathways, thus leading to the production of chemicals with interesting structures (Oliveira *et al.*, 2012). Moreover, compounds from natural resources possess high affinities towards target molecules

rendering them as effective drug candidates (Bhatnagar *et al.*, 2012). Worldwide, over 10,000 metabolites have been isolated from marine organisms mainly; sponges, soft corals, fungi and bacteria (Ebel, 2012). Due to the broad spectrum of chemical properties of marine natural products, they are known to exhibit a wide range of bioactivities including; antimicrobial, anticancer, anti-tuberculosis, antiviral, anti-parasitic, anti-helmintic, anti-malarial, anti-protozoal, anti-coagulant, anti-platelet, anti-inflammatory, anti-diabetic, and antitumor activities (Jones, 2008; Imhoff *et al.*, 2011).

Over the years, the search for natural products from marine organisms have evolved from merely isolation and description of natural compounds, to more intensive studies such as the investigation of their biological properties and most importantly, the biosynthetic pathways leading to the production of these natural compounds to ensure sustained production for pharmaceutical applications (Suyama *et al.*, 2011). A variety of marine organisms have been studied for natural products over the years, and they include; plants, invertebrates, algae, bacteria, fungi and protozoa (Gul *et al.*, 2005). Overall, marine derived fungi are now widely accepted as promising sources for the discovery of novel compounds with pharmaceutical properties. According to Bhatnagar *et al.* (2012), marine derived fungi are the major producers of antibacterial compounds among marine microbial flora. Keeping in mind the underestimated biodiversity of marine derived fungi, we can expect marine derived fungi to play a prominent role in the discovery of bioactive chemical compounds, to serve as potential drug candidates in line with the growing need of new natural products.

2.2 Marine derived fungi

Fungi are classified as lower eukaryotes and are also known as heterotrophic osmotrophs (Dick, 1997). They are incapable of synthesizing their own food but rather obtain nutrients directly from dead or living organisms or materials (Dick, 1997). Previously mistaken to be associated with the plant kingdom, modern biologists have now categorized them into their own kingdom known as "The fifth kingdom". Fungi were known to be diverse some 600 million years ago and have continued diversifying to such an extent that they are now classified into numerous groups (Dick, 1997). Worldwide, there are about 100,000 known fungal species and around 800 new species being named new to science every year (Jones *et al.*, 2012)

The 1991 hypothesis of having approximately 1.5 million fungal species on Earth by Hawksworth *et al.* (2011) is now considered an underestimate by many (Blackwell, 2011). Recent use of molecular methods in the classification of fungi has given rise to a new estimate of fungal diversity at about 5.1 million species (Blackwell, 2011). Fungi can range from microfungi such as molds and yeasts to macrofungi such as mushrooms and truffles. Certain types of macrofungi are considered delicacies and thus widely cultivated by humans for food supply. Microfungi can cause positive or negative impacts. Some microfungi are considered pathogenic and harmful to humans, animals and plants, while others are known to be producers of metabolites and enzymes proven beneficial to human life (Bennett, 1998).

Marine fungi are microorganisms that complete their entire life cycle in the sea whereby they can grow and sporulate exclusively in the marine habitat (Kohlmeyer *et al.*, 2003; Hooley *et al.*, 2006). According to Kohlmeyer *et al.* (2003), in a broad ecological definition, marine fungi are classified into two groups; obligate marine fungi, which are able to grow and sporulate exclusively in a marine habitat and secondly, facultative marine fungi, also known as marine derived fungi which are those from

freshwater or terrestrial habitat but is able to grow and possibly sporulate in the marine environment. Besides that, marine derived fungi can also be categorized based on their niche or in other words, the habitats and substrata that they occupy. Kohlmeyer *et al.* (1991) described four ecological groupings of fungi namely; lignicolous, arenicolous, algicolous and manglicolous fungi. Lignicolous fungi grow on submerged woody material mainly driftwood while arenicolous fungi are associated with sand grains. Follicolous fungi are found on leaves immersed or shed in the marine habitat while fungi isolated from submerged mangrove parts are called manglicolous fungi. Algicolous fungi are specific for fungi that occur on marine algae (Kohlmeyer *et al.*, 1991).

Buee *et al.* (2009) characterized functional ecological groups of fungi by linking fungal diversities to their niches in the environment. Currently, there are three functional ecological groups of fungi namely; parasitic, saprotrophic and mutualistic (Buee *et al.*, 2009). Parasitic fungi are fungi that depend on a host organism for nutrition and protection in turn, causing harm to the host organism. On the other hand, mutualistic fungi are fungi that are attached to a host organism where both fungi and host benefit from this association. Saprotrophic fungi are a group of fungi that are normally found on decaying matter such as plants or animals.

2.2.1 Natural products from marine derived fungi

Soil fungi and terrestrial plants have long been the focus as natural product reservoirs; however, chemical compounds isolated from these sources have been known to produce repeated chemical structures with common carbon backbones (Ebel, 2012). As the requirement for new therapeutic agents is ever increasing, scientists have now shifted focus upon diverse sources such as, thermophiles from ocean vents, soils subject to extreme radiation, endolithic fungi, marine organisms and endophytes (Jones *et al.*,

2008). The 1990's showed a drastic increase in marine microbial metabolites that continues until today, and marine derived fungi from various substrates have been recognized as a rich resource of novel bioactive metabolites with potential biomedical applications (Zhang *et al.*, 2008a). Figure 2.1 shows the percentage of new compounds from marine derived fungi based on the various substrates that they were isolated from. Based on Figure 2.1, it is noted that the highest percentage of new compounds were from the marine derived fungi isolated from algae, sponges and sediments including deep sea at 21%, 19% and 16% respectively.

Many metabolites from marine derived fungi have shown to possess antimicrobial, antifungal, cytotoxic, antitumor, immuno-stimulatory, antiviral and kinase inhibitory activity (Wu *et al.*, 2008). Reports suggest that the majority of marine natural products have novel pharmaceuticals values (Davidson, 1995; Liberra *et al.*, 1995; Jensen *et al.*, 2002; Bringmann *et al.*, 2005). The groups of chemical compounds of marine fungal origin are divided into seven groups namely; polyketides, alkaloids, terpenoids, peptides, prenylated polyketides, shikimates and lipids. According to Figure 2.2, the majority of marine derived fungi secondary metabolites are from the chemical group polyketides and alkaloids groups. Some of the reported secondary metabolites of marine derived fungi origin are listed in Table 2.1 while selected chemical compounds are shown in Figure 2.3.

It is believed that due to harsh environmental factors, marine derived fungi are expected to produce high numbers of active secondary metabolites with unique structures (Vongvilai *et al.*, 2004). A study by Pan *et al.* (2008) reported the discovery of 42 new compounds and 35 known compounds from marine derived fungi isolated from South China Sea. To date, more than 500 new natural products have been isolated from marine derived fungi (Rateb *et al.*, 2010). The first report of a bioactive natural product from a marine derived fungus dates back to the 1940s when cephalosporin C

was isolated from the fungus *Acremonium chrysogenum* Gams collected from a sewage outlet in the Mediterranean Sea (Proksch *et al.*, 2008). Cephalosporin C is the precursor chemical compound of modern cephalosporin antibiotics (Proksch *et al.*, 2008). Existing modern drugs of fungal origin include b-lactam antibiotics, griseofulvin, cyclosporine A, taxol, ergot alkaloids, and lovastatin (Suryanarayanan *et al.*, 2009). On-going research on the secondary metabolite chemistry of marine derived fungi has led to the discovery of numerous novel compounds with promising pharmacological potential such as those with anti-cancer and anti-microbial properties (Proksch *et al.*, 2008). There are numerous marine derived fungi reported to produce compounds with interesting bioactivities. One such example is the marine derived fungus *Aspergillus* sp. which has been widely known to produce many novel chemical compounds such as azonazine with anti-inflammatory activities, spirotryprostatin with phytoregulating activities and the anti-cancer compound, diphenly (Liu *et al.*, 2006; Ebel, 2010; Afiyatullov *et al.*, 2012). Kitano *et al.* (2012), reported the discovery of a new anti-tumour metabolite, pileotin A from *Aspergillus* sp isolated from a sea urchin. The marine derived fungi *Gymnascella* sp. has also been widely studied over the years and has been known to produce Gymnastatins, Gymnasterones and Dankastatins with promising anti-tumour and cytostatic activities (Amagata *et al.*, 1998; Amagata *et al.*, 2008). Extensive research has also been carried out to explore the antimicrobial properties of fungal secondary metabolites to cope with the ever increasing occurance of microbial drug resistance. A study by Gallordo *et al.* (2006), reported the isolation of amides of D-*allo* and L-isoleucine derivative with antibacterial and antifungal properties from the marine derived fungi *Acremonium furcatum*. A recent study by Caballero *et al.* (2013) reported the production of toluquinol, a strong inhibitor of angiogenesis thus making this compound a promising drug candidate for further evaluation to treat cancer and other angiogenesis-related conditions.

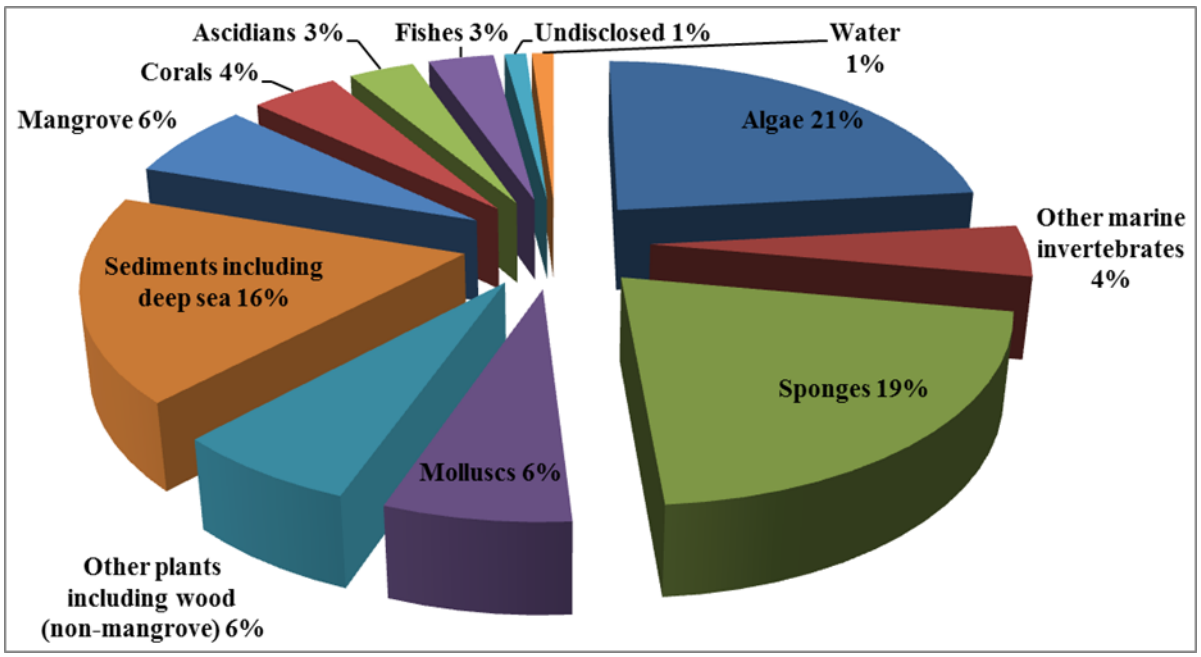


Figure 2.1: Percentage of new compounds from marine derived fungi with respect to the fungi source and substrates (Ebel, 2012)

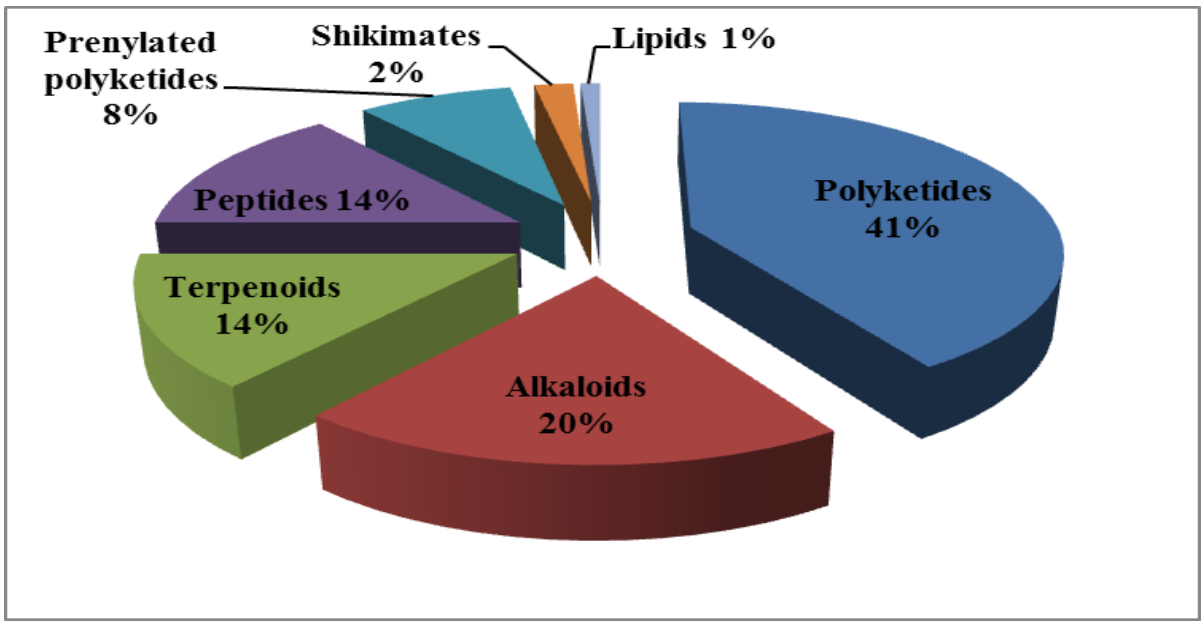


Figure 2.2: Percentage of new compounds from marine derived fungi with respect to their chemical grouping (Ebel, 2012)

Table 2.1: Secondary metabolites from selected marine derived fungi

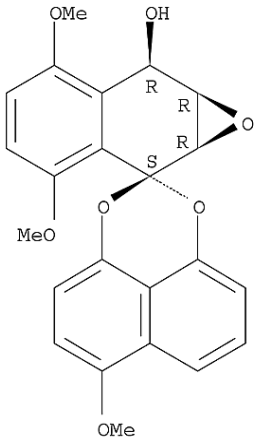
Fungi identified	New compound	Bioactivity	Reference
Penicillium sp.	Penicillazine	Antibacterial	Iwatsuki *et al.*, 2011
	Cephalosporolides H-I		Li *et al.*, 2007
	Biverlactones A-D		Bringmann *et al.*, 2010
	Sorbifuranones A-C		
	Sorbicillinoid		
Penicillium citrinum	Benzopyranones	Antimicrobial	Khamthong *et al.*, 2012
	Isochroman	Antimicrobial	
	Anthraquinone-citrinin derivatives	Antimicrobial	
	Citrin derivatives	Anticancer	Chen *et al.*, 2011
	Penicitrinols C-E	Anticancer	
Penicillium aurantiogriseum	Verrucisidinol	Antimicrobial	Yu *et al.*, 2010a
	Verrucosidinol acetate	Antimicrobial	
Penicillium commune	Comazaphilones A-F	Antimicrobial, Anticancer	Gao *et al.*, 2011
Xylaria sp.	Xyloallenolide A		Lin *et al.*, 2001
	Xyloketals A-H		Liu *et al.*, 2006
Paecilomyces sp.	Paeciloxanthone	Antibacterial, Anticancer	Wen *et al.*, 2008
Eutypella scoparia	7,8-dihydroxy-3,5,7-trimethyl-8,8a-dihydro-1H-isochromen-6(7H)-one	Anticancer	Li *et al.*, 2012
	6-(hydroxymethyl)-2,2-dimethyl-3,4-dihydro-2H-chromene-3,4-diol	Anticancer	
Stysanus-like sp.	Hypoxylin A-B		Wang *et al.*, 2005
Tryblidiopycnis sp.	Chloro-monoterpene		Huang *et al.*, 2006
Guignardia sp.	Vermistatin	Anticancer, Antifungal	Xia *et al.*, 2007
Ascochyta sp.	Ascochytatin	Antibacterial	Kanoh *et al.*, 2008a
	Ascosalipyrrolidinone A	Antimicrobial	
	Ascospiroketals A-B		
Zygosporium sp.	Sulfoalkylresorcinol	Antibacterial	Kanoh *et al.*, 2008b
Phomopsis sp.	Phomolide B	Antibacterial	Du *et al.*, 2008
Nodulisporium sp.	Nodulisporacid A	Antimalarial	Kasettrathat *et al.*, 2008
Chaetomium sp.	Chaetoxanthone B	Antimalarial, Antiprotozoal	Pontius *et al.*, 2008a
	Chaetominedione	Anticancer	Abdel-Lateff, 2008

Table 2.1, continued

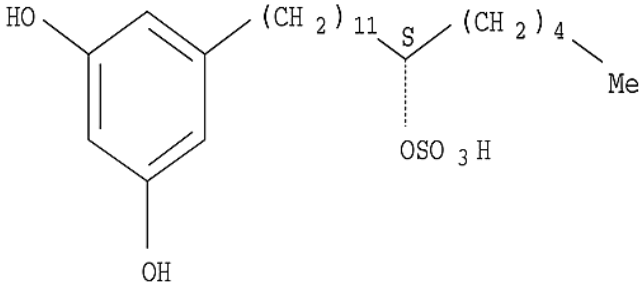
Chaetomium globosum	Chaetoglobosin Fex	Anti-inflammatory	Dou *et al.*, 2011
	Cytoglobosins A-G	Anticancer	Cui *et al.*, 2010
Aspergillus insulicola	Azonazine	Anticancer	Wu *et al.*, 2010
Aspergillus sulphureus	Decumbenone C	Anticancer	Zhuravleva *et al.*, 2012
Aspergillus carbonarius	8'-O-Demethylnigerone	Anti-tuberculosis	Zhang *et al.*, 2008a
	8'-O-Demethylisonigerone	Anti-tuberculosis	
Periconia sp.	Macrosphelide M	Xanthine oxidase inhibition	Yamada *et al.*, 2007
	Peribysin J		
Aigialus parvus	Aigialomycins A-E	Antimalarial, Anticancer	Isaka *et al.*, 2002
	Hypothemycin	Anticancer	
	Aigialone	Anticancer	Vongvilai *et al.*, 2004
	Aigialospirol	Anticancer	
Exophiala sp.	Circumdatin I	Ultraviolet protecting	Zhang *et al.*, 2008b
Lignincola laevis	7-hydroxyergosterol	Anticancer	Abraham *et al.*, 1994
Monodictys putredinis	Monodictysin C	Anticancer	Krick *et al.*, 2007
	Monodictyochromes A-B	Anticancer	Pontius *et al.*, 2008b
	Monomeric xanthones	Anticancer	
Verruculina enalia	Enalin A	Antimicrobial, Anti-diabetic	Lin *et al.*, 2002
Halorosellinia oceanica	2-hexylidene-3-methylsuccinic acid	Anticancer, Antimalarial	Chinworrungsee *et al.*, 2001
	Cytochasin	Anticancer	
	5-carboxymellein	Anticancer, Antimalarial	
	Halorosellins A-B	Antimalarial, Antimycobacterial	Chinworrungsee *et al.*, 2002
	Hypoxylin A-B		
	Sesquiterpenoid lactone		
Acremonium sp.	Acremolides A-D	Unknown	Ratnayake *et al.*, 2008
	Lipodepsipeptides		
Acremonium striatisporum	Diterpenic altrosides	Anticancer	Afiyatullov *et al.*, 2000
Acremonium furcatum	Amides of D-allo	Antimicrobial	Gallardo *et al.*, 2006
	Amides of L-isoleucine	Antimicrobial	
Acremonium strictum	Acremolin	Anticancer	Julianti *et al.*, 2012
	Acremostrcitin	Antibacterial	Julianti *et al.*, 2011

Table 2.1, continued

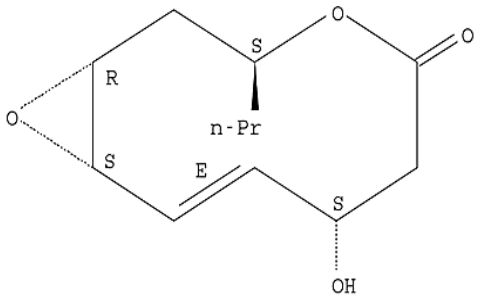
Gymnascella sp.	Gymnastatins Q-R	Anticancer	Amagata *et al.*, 2008
	Dankasterone	Anticancer	
	Dankastatins A-B	Anticancer	
	Gymnasterone	Anticancer	Amagata *et al.*, 1998
Gymnascella dankaliensis	Gymnastatins A-C	Anticancer	Numata *et al.*, 1997
	Gymnastatins F-H	Anticancer	Amagata *et al.*, 2006
Aspergillus sp.	Diphenyl ether	Anticancer	Liu *et al.*, 2006
	Pileotin		Kitano *et al.*, 2012
Aspergillus fumigatus	Spirotryprostatin	Phytoregulating	Afiyatullov *et al.*, 2012
Fusarium sp.	Fusaranthraquinone	Antimicrobial,	Trisuwan *et al.*,
	Fusarnaphthoquinones A-C	Antimycobacterial, Antimalarial,	2010
	Fusarone	Anticancer	
Deuteromycete sp.	Deuteromycols A-B	Antibacterial	Nawwar *et al.*,
	Benzofuranoids	Antibacterial	2010
Phoma herbarum	Arthropsadiol C	Neuraminidase inhibitory	Zhang *et al.*, 2012
	Massarilactone H	Neuraminidase inhibitory	
Botryotinia sp.	Botcinin	Anti-diabetic	Kim *et al.*, 2012



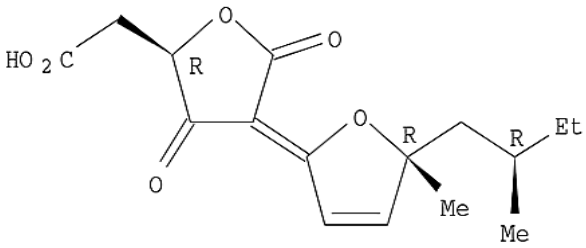
Ascochytatin



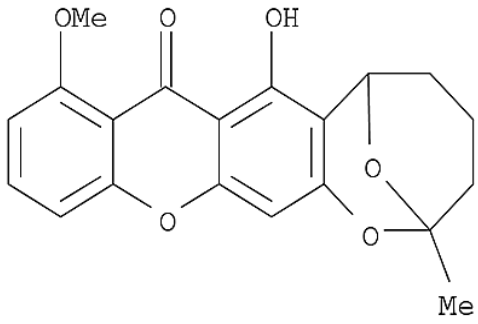
Sulfoalkylresorcinol



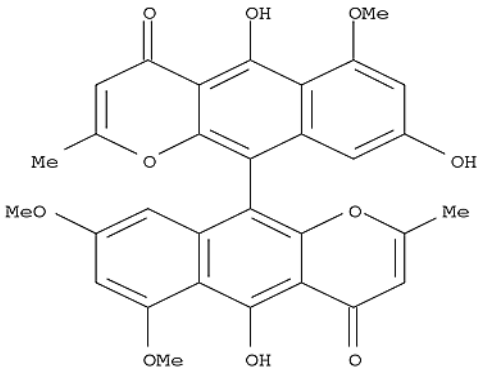
Phomolide B



Nodulisporacid A

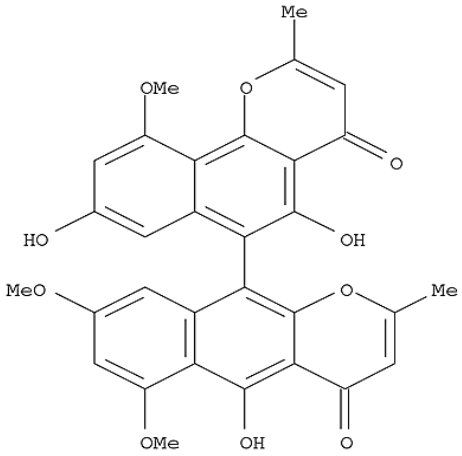


Chaetoxanthone B

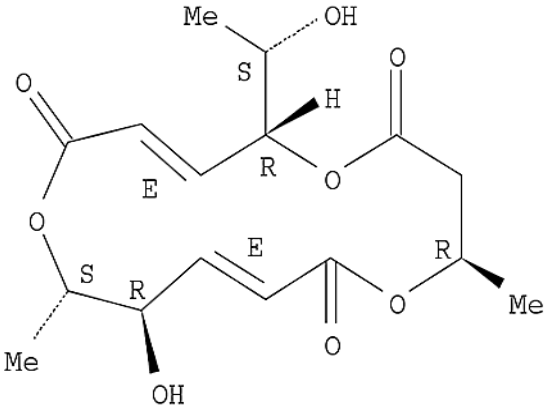


8’-O-Demethylnigerone

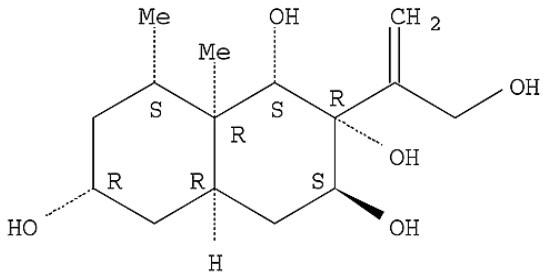
Figure 2.3: Secondary metabolites from selected marine derived fungi



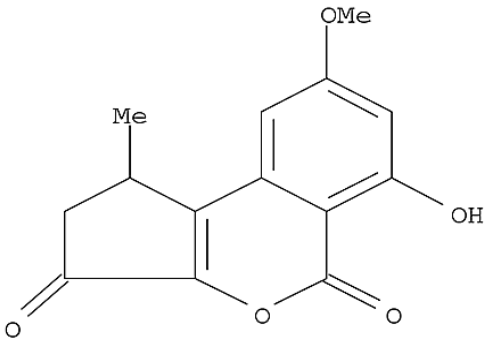
8'-O-Demethylisonigerone



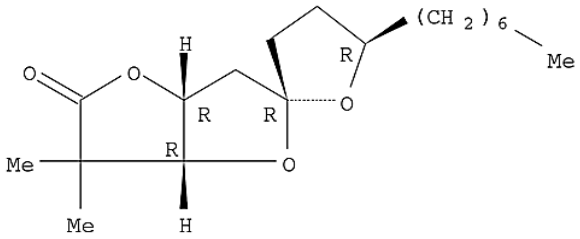
Macrosphelide M



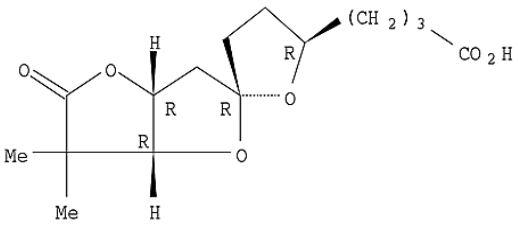
Peribysin J



1-Deoxyrubralactone

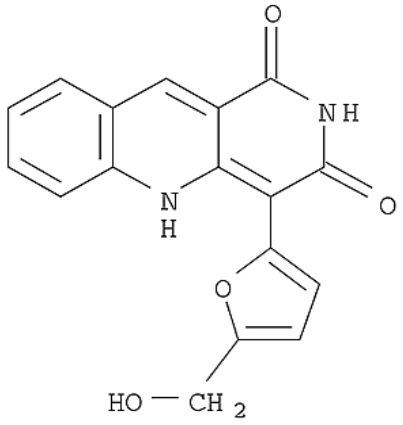


Cephalosporolides H

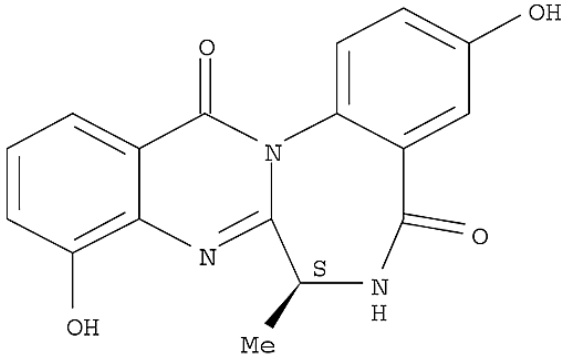


Cephalosporolides I

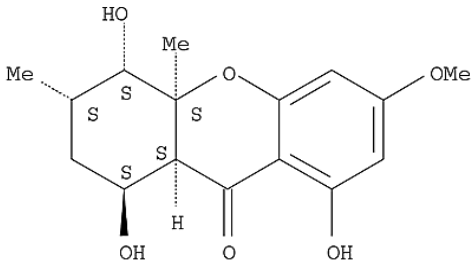
Figure 2.3: Secondary metabolites from selected marine derived fungi (continued)



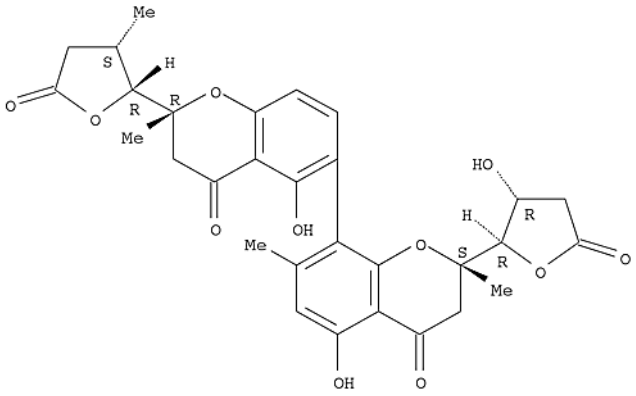
Chaetominedione



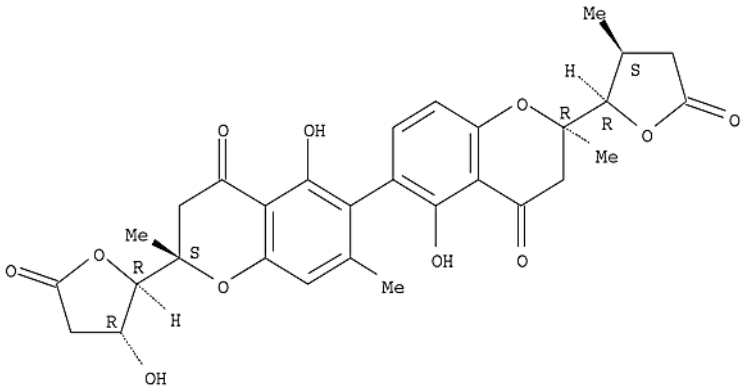
Circumdatin I



Monodictysin C



Monodictyochrome A



Monodictyochrome B

Figure 2.3: Secondary metabolites from selected marine derived fungi (continued)

2.2.2 Antibacterial compounds from marine derived fungi and their mechanisms

The first antibacterial compound isolated from an obligate marine fungi was cephalosporin C from the fungi *Cephalosporium acremonium* (now named *Acremonium chrysogenum*) (Bugni *et al.*, 2004). Cephalosporin C is the precursor molecule that led to the development of modern day cephalosporins that are used in the treatment of infections caused by Gram positive and Gram negative bacteria (Liu *et al.*, 2011). Cephalosporins belong to the beta lactam class of antibiotics similar with penicillin, in which they possess the beta-lactam ring (Liu *et al.*, 2011). Cephalosporins are bacteriocidal in their mode of action, whereby they interfere with the synthesis of the bacterial cell wall (Liu *et al.*, 2011). The proper formation of the cell wall is essential for the growth of the bacteria and peptidoglycan is an important component in the cell wall that provides stability through its cross-linked latticework structure (Singh *et al.*, 1999). Beta-lactam antibiotics generally inhibit the completion of the cross-links by the membrane bound transpeptidase enzyme thus killing the bacteria (Singh *et al.*, 1999).

The majority of new compounds from marine derived fungi are polyketides, which also encompasses the antibiotic class macrolides (Ebel, 2012). There have been reports on the isolation of antibacterial macrolides from marine derived fungi (Isaka *et al.*, 2002; Shiomi *et al.*, 2002). Macrolides are a group of drugs with the presence of the macrolide ring and possesses bacteriostatic activities (Shiomi *et al.*, 2002). Macrolides are protein synthesis inhibitors, whereby they inhibit the biosynthesis of bacterial protein by binding to the large ribosomal subunit, close to the peptidyl transferase center thus preventing the formation of peptide bonds during protein elongation (Tenson *et al.*, 2003). Alkaloids are the second major fraction of new metabolites from marine derived fungi (Ebel, 2012) with reports that certain alkaloids produced by these organisms possesss antibacterial activities (Li *et al.*, 2006; Du *et al.*, 2012). Studies have reported that antibacterial alkaloids exert its antibacterial activity by inhibiting the

transpeptidase enzyme sortase A, thus interrupting bacterial cell wall formation (Mayer *et al.*, 2013). Marine derived fungi are also known to produce antibacterial diterpenes, a type of terpene (Gao *et al.*, 2011). Although the mechanism of action of diterpenes from marine derived fungi have not been extensively studied upon, Ghosh *et al.* (2013) stated that diterpenes with antibacterial activities usually exert their action by causing significant bacterial cell membrane damage leading to cell leakage.

2.3 Manglicolous and endophytic marine derived fungi as potential natural product reservoirs

2.3.1 Manglicolous fungi

Mangroves are wetland forests located along the intertidal areas of estuarine sea coasts and river mouths in tropical and subtropical parts of the world (Logesh *et al.*, 2012). It is believed that 25% of the world's tropical coastlines across 112 countries, covering an approximate area of 150,000-181,000 km^2 are dominated by mangroves (Sridhar *et al.*, 2012). The mangroves of Malaysia ranks 6^{th} largest in the world with a total coverage area of 6458.5 km^2, of which 1097.95 km^2 is in Peninsular Malaysia and 5360.55 km^2 is located in East Malaysia (Spalding *et al.*, 2010). Mangroves are known to be one of the most productive ecosystems with intense carbon recycling, thus making them excellent harbors of fungi lifeforms (Arfi *et al.*, 2012).

Manglicolous fungi can be classified into five main groups namely; terrestrial, phylloplane, parasites, saprobes and endophytes (Sridhar *et al.*, 2012). Manglicolous fungi are commonly found on submerged areas of mangroves, growing on substrates such as wood, leaf, seagrass, sediments, soil, sand, algae, coral and calcareous materials and they are considered the second largest group of marine fungi (Hyde, 1990).

The role of fungi in the ecosystem of the mangrove is considered vital as they act as decomposers of organic matter (Sridhar *et al.*, 2012). The first manglicolous fungus was reportedly isolated from Australia by Cribb *et al.* (1955). All major fungi taxa namely; Ascomycota, Basidiomycota, Deuteromycota, thraustochytrids and lower fungi, can be found in the tropical and sub-tropical mangroves (Jones *et al.*, 1997). The unique characteristics of the mangroves such as semi-saline surroundings with periodic flooding, supports an array of diverse microorganisms with the ability to produce novel compounds due to their interactions with their habitat (Amrani *et al.*, 2012).

Marine derived fungi associated with mangrove plants such as *Rhizophora mucronata*, *Rhizophora apiculata* and *Avicenna officinalis* have been reported to produce new and and novel compounds beneficial to medicine and agrochemicals (Bhimba *et al.*, 2011; Rukachaisirikul *et al.*, 2012). Pan *et al.* (2008) and Li *et al.* (2010) reported that the mangrove habitat along the South China Sea coastline has served as a reservoir for marine derived fungi with novel secondary metabolites.

2.3.2 Host plant and endophytic fungi

Plant scientists believed that there may be an unaccounted number of organisms known as endophytes that reside within the inter-cellular spaces of plant tissue (Strobel, 2003). Endophytes are defined as microorganisms (bacteria or fungi) that reside inside the tissues of an otherwise healthy plant also known as the host plant (Strobel, 2003). The symbiotic relationship between an endophytic fungi and its host plant holds a major role in structuring plant communities by affecting colonization, competition, coexistence and soil nutrient dynamics (Borges *et al.*, 2009). During this symbiotic relationship, neither the host plant nor the fungi is harmed in any way. The fungi benefits by gaining protection and nutrients from the plant, in return the fungi produces certain secondary metabolites that benefits the host plant and protects it from pathogens and herbivores (Kusari *et al.*, 2012).

Scientists have come up with a theory that links these long term host-endophyte interactions to the development of specialized genetic systems in the endophytes to facilitate the transfer of information from host plant to endophyte. This way, there is an exchange of biochemical pathways allowing the endophytes to produce compounds similar or common to their host plant (Borges *et al.*, 2009). These secondary metabolites, harbors diverse therapeutic activities applicable in medicine and there have been instances where the fungi is able to produce certain chemical compounds similar to

its host; a known example is the compound taxol, originally isolated from the Pacific Yew tree which was also isolated from the endophytic fungi *Taxus andreanae* (Radic *et al.*, 2012). Studies have reported that in comparison to novel compounds from soil microflora, 51% of compounds isolated from endophytic fungi were previously unreported (Strobel, 2003). Radic *et al.* (2012) stated that the number of secondary metabolites reported from endophytic fungi is larger than that from any other endophytic microorganism. However, this may also be due to the frequency of isolation of endophytic fungi from plants (Radic *et al.*, 2012).

According to Yu *et al.* (2010b), in order to obtain endophytic fungi prone to producing novel compounds, there are several criterias to consider when selecting the endophyte host plant namely; plants in areas of high biodiversity, plants in harsh or challenging habitats, plants surrounded by pathogen infected plants, plants with traditional medicinal values and lastly plants occupying a certain ancient land mass.

Research shows that there are over 300,000 plant species on earth, and it is estimated that there may be one or more endophytic fungi residing in each individual plant. However over the one million species of endophytic fungi estimated, only a small fraction has been described and recorded (Yu *et al.*, 2010b). With regards to the estimated abundance of plant endophytic fungi yet to be explored and the ever increasing number of novel and interesting compounds isolated from these fungi, it is justified that these group of fungi can be untapped reservoirs of unique and biologically potent compounds with high commercial potential (Shiono *et al.*, 2011; Cheng *et al.*, 2012).

2.4 Bacterial resistance and nosocomial infections

More than 30% of the world's populations are likely affected by bacterial infections with roughly two million fatalities recorded each year (Monaghan *et al.*, 2006). Antibiotic resistance is fast emerging as a threat to the success of medical interventions of health care, at local, national and even global dimensions thereby creating clinical and therapeutic challenges to human health (Monaghan *et al.*, 2006). When continuously exposed to antibiotic compounds especially in the hospital environment, bacteria that are generally considered as normal human flora tend to develop significant antibacterial resistance to the antibiotics (Grundmann *et al.*, 2011). Most of these bacteria are truly opportunistic pathogens, thus the segment of society most vulnerable are usually the young, elderly and immune-compromised individuals (Grundmann *et al.*, 2011). The European Centre for Disease Prevention and Control (ECDC) rated antimicrobial resistance as one of the major infectious disease threats in Europe due to increasing levels of multidrug-resistance bacteria. A study by Zaidi *et al.* (2005), reported that 70% of hospital acquired neonatal infections could not be treated successfully with the drug regimen recommended by the World Health Organization (WHO). The development of new antibacterial agents against these multidrug-resistant bacteria is therefore regarded as crucial to public health need (Moran *et al.*, 2011). Nosocomial infection is defined as an infection acquired in the hospital by a patient whose illness upon admission was independent of the infection. The timeline of infection is within 48 hours of hospital admission, 3 days of discharge or 30 days of surgery (Inweregbu *et al.*, 2005). The WHO reported that at any one time, over 14 million people worldwide suffer from complication due to nosocomial infections. Nosocomial infections occur worldwide affecting both developed and poor countries causing significant burden to both patient and public health (World Health Organization, 2002; Centers for Disease Control, 2013)

2.4.1 Characteristics of test bacteria and their relation to nosocomial infections

2.4.1.1 Staphylococcus aureus

Staphylococcus aureus are Gram positive, non-motile, catalase positive, spherical shaped bacterium (cocci) (Hait, 2012). Upon microscopic examination, these microorganisms appear either in pairs, short chains or in a bunch like grape cluster (Hait, 2012). This bacterium is commonly found in soil, water, air, and also possess the ability to live in humans and animals (Hait, 2012). They are one of the most resistant non-spore-forming human pathogens which are able to survive in dry states for long (Hait, 2012).

Research has shown that approximately 20% if the human population carry this bacterium in their nasal cavity (Weidenmaier *et al.*, 2012). Although commonly acting as a commensal, this bacterium can also evolve into a human pathogen triggering a wide range of acute and chronic diseases (Weidenmaier *et al.*, 2012). *Staphylococcus aureus* is the predominant agent associated with staphylococcal food poisoning; where they produce enterotoxins that are fast acting and usually is not destroyed by the heat introduced during cooking of contaminated food (Gadepalli *et al.*, 2009).

Staphylococcus aureus is the most frequently isolated microorganism in skin and soft tissue infections (SSTIs), which is the common cause of morbidity in hospitalized patients around the world (Gadepalli *et al.*, 2009). However the development of antimicrobial resistance has further complicated treatment management of these infections (Gadepalli *et al.*, 2009). Some of of the other diseases associated with this bacterium are; toxic shock syndrome, pneumonia, postoperative wound infection and nosocomial bacteremia (Hait, 2012).

Staphylococcus aureus is fast becoming resistant to standard commercial antibiotics with the emergence of methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) (Joo *et al.*, 2012). Reports show that MRSA is

the main cause of healthcare associated infection with a record number of two to fifty-three million human carriers worldwide and a mortality rate of about 20% (Joo *et al.*, 2012).

2.4.1.2 Bacillus cereus

Bacillus cereus is a Gram positive, motile, rod-shaped bacterium with haemolytic activity and is able to form heat resistant endospores (Tallent, 2012). They are classified as saprobes and are thus widespread in the environment, commonly found in soil and plants (Tallent, 2012). The main disease associated with *B. cereus* is food poisoning caused by the necrotic enterotoxin produced by this bacterium when contaminated food are consumed (Lee *et al.*, 2009; Tallent, 2012). Although cooking can kill the bacterium, the toxin might still be active (Tallent, 2012).

There are two ways this bacterium can induce food poisoning, firstly the production of toxins in the intestine when contaminated food is ingested and secondly, *B. cereus* can also produce a different kind of toxin in contaminated food especially in starchy foods like rice (Tallent, 2012). The toxins produced by this bacterium are also responsible for invasive infections such as panopthalmitis, pneumonia, endocarditis, meningitis or septicaemia, especially in immunocompromised patients (Dubouix *et al.*, 2005). *Bacillus cereus* has been listed as one of the foodborne microorganisms of public health significance and foodborne illnesses (Lee *et al.*, 2009).

2.4.1.3 Bacillus subtilis

Bacillus subtilis is a Gram positive, catalase positive, rod-shaped bacterium that is able to form tough, protective endospores (Bridier *et al.*, 2012). This bacterium is commonly found in the soil and is rarely associated with human infections in contrary to *B. cereus* (Bridier *et al.*, 2012). However, recent research by Bridier *et al.* (2012), reported the

isolation of an antimicrobial resistant *B. subtilis* from an endoscope washer-disinfector in the hospital environment. The *B. subtilis* strain isolated in the study by Bridier *et al.* (2012) was able to produce a thick immersed biofilm with specific protruding structures and displayed protective characteristics towards *S. aureus*. The finding by Bridier *et al.* (2012) could serve to prove that although never once thought to be pathogenic, *B. subtilis* may be emerging as a causal agent in nosocomial infections and may in fact aggravate the effects primarily caused by other nosocomial pathogens.

2.4.1.4 Micrococcus luteus

Micrococcus luteus is a Gram positive, spherical (cocci) bacterium that are commonly found on the human skin and mucosal membranes of otherwise healthy individuals and have been rarely associated as human pathogens (Oprica, 2009). Although considered to be part of the normal flora of the skin, *M. luteus* is still noted as a contaminant when isolated from clinical specimens (Oprica, 2009). This bacterium is an opportunistic pathogen normally affecting immune-compromised individuals causing diseases such as bacteraemia and sepsis, endocarditis, pneumonia, peritonitis, central nervous system infection, and traumatic and post-operative endophthalmitis (Miller *et al.*, 2007). In the hospital environment, this bacterium has been closely associated with indwelling intravenous lines and dialysis among others (Altuntas *et al.*, 2004).

2.4.1.5 Enterococcus faecalis

Enterococcus faecalis is a Gram positive, catalase-negative, spherical and ovoid bacterium that usually occurs singly, in pairs or in chains (Zhang *et al.*, 2012). *Enterococcus faecalis* is usually a commensal in human and animal gastrointestinal tracts and thus usually recovered from faeces and clinical specimens (Zhang *et al.*, 2012). This bacterium is an opportunistic pathogen and is able to tolerate harsh

conditions producing virulence factors such as, cytolysin/hemolysin and adhesins among others (Gil *et al.*, 2009). The toxin cytolysin produced by some *E. faecalis* strains display both haemolytic and bactericidal activities, which upon ingestion, may cause diarrhoea, cramps, nausea, vomiting, fever and chills in otherwise healthy individuals; thus giving this bacterium an added advantage to survive in extreme conditions and eliminate competition (Zhang *et al.*, 2012). The people most likely to suffer serious problems are usually those with underlying illnesses and immune-compromised individuals (Zhang, *et al.*, 2012).

The emergence of vancomycin-resistant and multi-drug resistant enterococci strains poses a global threat to human health and may possess serious impact on human healthcare (Gil *et al.*, 2009). In the hospital environment, this bacterium has been reported to be the frequent cause of endocarditis, bacteraemia, urinary and wound infections (Gil *et al.*, 2009). Furthermore, studies by Stuart *et al.* (2006) reported that *E. faecalis* is the main cause of asymptomatic, persistent endodontic infections ranging from 24% to 77%.

2.4.1.6 Escherichia coli

Escherichia coli is a Gram negative, rod-shaped bacterium predominantly found as a commensal in the human gut (Wu *et al.*, 2012). Although commonly associated as normal intestinal flora, there are six pathogenic groups namely; entero-toxigenic *E. coli* (ETEC), entero-pathogenic *E. coli* (EPEC), entero-hemorrhagic *E. coli* (EHEC), entero-invasive *E. coli* (EIEC), entero-aggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC) (Feng, 2012). ETEC has been known to cause travellers' diarrhoea with the World Health Organization (WHO) attributing 380,000 deaths each year, mainly among children (Feng, 2012). Infantile diarrhoea is usually associated with EPEC with mortality rates ranging from 25% to 50% (Feng, 2012). One of the more harmful *E. coli*

strain is the EHEC which produces Shiga toxins (Feng, 2012). Infections of EHEC can range from mild diarrhoea to more severe diseases such as haemorrhagic colitis causing severe abdominal cramps and bloody diarrhoea which may progress to life-threatening complications such as, thrombotic thrombocytopenia purpura (TTP) (Feng, 2012). The use of antibiotics for EHEC infections has proven to have more harm than benefits with speculation that the antibiotics lyse EHEC cells thus releasing more Shiga toxin into the infected host (Feng, 2012). EIEC is on the other hand a less harmful strain commonly causing mild bacillary dysentery with the Centre's for Disease Control and Prevention (CDC) listing a death rate of zero for infections by this bacterium (Feng, 2012).

Escherichia coli is also a common pathogen associated with nosocomial urinary tract infections (UTIs) (Bonkat *et al.*, 2011). The emergence of extended-spectrum beta-lactamase *E. coli*, a strain resistant to all penicillins, first-, second- and third generation cephalosporin sans aztreonam has become an on-going problem in the hospital environment (Bonkat *et al.*, 2011).

2.4.1.7 Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram negative, monoflagellated, rod-shaped bacterium that are commonly found in the soil, water and also on humans and animals (Iversen *et al.*, 2008). It is an opportunistic pathogen and usually affects immune-compromised individuals (Iversen *et al.*, 2008). This bacterium is one of the leading causes of nosocomial infections, whereby almost 10% of hospital acquired infections are caused by *P. aeruginosa* and is ranked among the top ten causes of bacteraemias in hospitals (Iversen *et al.*, 2008).

Pseudomonas aeruginosa are known to be associated with several hospital acquired infections ranging from urinary tract infection, respiratory and central nervous system infections to ear, eye, bone, and skin infections (Iversen *et al.*, 2008; Bilavsky *et*

al., 2013). A severe complication associated with this bacterium is blood stream infections which carries a mortality rate ranging from 18% to 39% (Suarez *et al.,* 2009). *Pseudomonas aeruginosa* has been notoriously known to acquire and develop resistance to most standard antibiotics thus increasing the mortality rates of its infections (Suarez *et al.,* 2009).

CHAPTER 3

MATERIALS AND METHODS

3.1 Collection, isolation and identification of marine derived fungi

3.1.1 Endophytic fungi of marine associated host plants

The two marine associated plants chosen in this study were, *Vitex rotundifolia* and *Ipomoea pes-caprae* as shown in Figure 3.1. *Vitex rotundifolia* was collected from Kijal Beach, Terengganu (east coast, Peninsular Malaysia), while the plant *I. pes-caprae* was collected from Port Dickson Beach, Negeri Sembilan (west coast, Peninsular Malaysia).

Vitex rotundifolia, commonly known as Beach *Vitex,* is an exotic deciduous woody shrub that does not grow high but rather forms thick patches on the soil (Maddox *et al.*, 2007). Beach *Vitex* can reach one to two inches high and around twelve inches in diameter but their stem runners can grow up to 60 inches long (Maddox *et al.*, 2007). This fast growing woody vine is able to tolerate the harsh conditions of beach sand dunes whereby, they can tolerate the drough conditions of the sand on the beach as well as the salt spray from the ocean (Maddox *et al.*, 2007). Beach *Vitex* is native to Malaysia, Indonesia and Hawaii, but is also found in China, Taiwan, Japan, India, Sri Lanka, Mauritius, Australia and the Pacific Islands (Maddox *et al.*, 2007).

Ipomoea pes-caprae is a pantropical, perennial, trailing vine with pick flowers (Devall, 1992). It known to be a widely distributed beach plant, forming wide and thick continuous mats on the beach sand dunes mostly in the tropical and subtropical areas in the world (Devall, 1992).

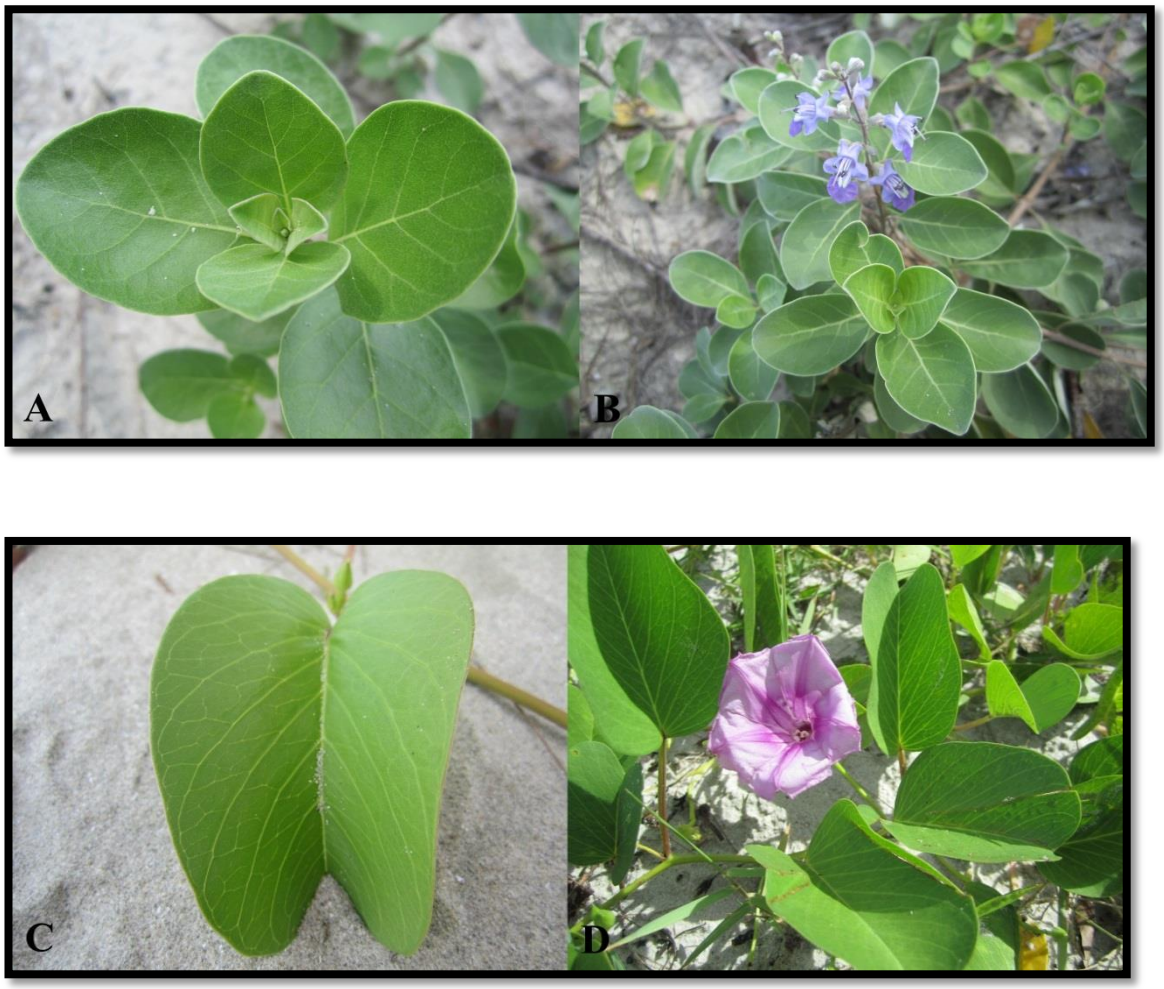


Figure 3.1: Photographs of endophytic fungi host plants. A and B: *V. rotundifolia*; C and D: *I. pes-caprae*

3.1.1.1 Collection and isolation of marine derived endophytic fungi

Whole plant materials were excised using clean scissors and placed in sterile polythene bags and transported back to the laboratory within four hours for analysis. Samples were washed with distilled water to remove surface sand and debris. The leaves and stem of the plant were then cut into standard 6 mm sizes and surface sterilized using a regimen of, 70% ethanol for two minutes followed by 4% bleach for one minute after which they were treated again with 70% ethanol for one minute and finally rinsed thrice in distilled water.

The surface sterilized plant materials were then placed on potato dextrose agar (PDA) and incubated at 37°C. The agar was observed daily for mycelia sporulation; germinating mycelia was then transferred to fresh media using a fine needle to obtain pure fungi cultures. The cultures were then transferred to agar slant and stored at 8°C until further use. The identification of the endophytic fungi was done using molecular methods as elaborated further in this dissertation.

3.1.2 Manglicolous fungi

The manglicolous fungi strains used in this study were obtained from the Institute of Biological Science (ISB) culture collection. All fungi strains were from the mangrove habitats of Peninsular Malaysia. The manglicolous fungi were maintained on PDA supplemented with 25 ppm marine salt.

Table 3.1: List of manglicolous fungi in the present study

Fungi	Culture collection number
Corollospora maritima Werderm.	ISB007
Dactylospora haliotrepha (Kohlm. & E. Kohlm.) Hafellner	ISB001
Dactylospora haliotrepha	ISB002
Dactylospora haliotrepha	ISB003
Dactylospora haliotrepha	ISB004
Fusarium sp.	ISB008
Henningsomyces sp.	ISB009
MF 28	ISB010
Saccardoella rhizophorae K.D. Hyde	ISB011
Verruculina enalia (Kohlm.) Kohlm. & Volkm.-Kohlm.	ISB005
Verruculina enalia	ISB006

3.2 Molecular identification of endophytic marine derived fungi

3.2.1 Deoxyribonucleic acid (DNA) extraction from marine derived endophytic fungi

Extraction of DNA was carried out using the "Plant Mini DNeasy Kit" from Qiagen. Firstly the samples were homogenized using a mortar and pestle. Secondly, 400 µl of Lysis buffer and 4 µl of RNase A were added to the samples, after which the samples were vortexed and incubated for ten minutes at 65°C. Next, 130 µl Precipitation Buffer was added and mixed with the sample, followed by 5 minutes incubation period on ice. The lysate was then centrifuged for five minutes at 14,000 rpm. The lysate was further pipetted into a QIAshredder Spin Column placed in a 2ml collection tube and centrifuged for two minutes at 14, 000 rpm. The flow-through was then transferred into a new tube without disturbing the pellet and 1.5 volumes of Washing Buffer was added and mixed. About 650 µl of the mixture was then transferred into a DNeasy Mini spin column placed in a 2ml collection tube and centrifuged for one minute at 9, 000 rpm. The flow through was discarded. The spin column was then transferred into a new 2 ml collection tube and 500 µl of Washing Buffer was added followed by centrifugation for one minute at 9000 rpm. The flow through was discarded and another 500 µl of Washing Buffer was added followed by centrifugation for two minutes at 14, 000 rpm. The spin column was then transferred to a new 2 ml centrifuge tube. Lastly, 100 µl of Elution Buffer was added for elution and incubated for five minutes at room temperature (15-25°C) and centrifuged for one minute at 9, 000 rpm.

3.2.2 Preparation of agarose gel

The agarose used in this study was 10 % agarose which was prepared by dissolving 1 g of agarose powder in 100 ml of distilled water. The slurry mixture was then heated in a microwave until it turned clear. The mixture was then left to cool for about ten minutes, after which 5 µl of the staining dye Cyber Safe was added and mixed well. The agarose mixture was then poured into the gel blocks and left to solidify.

3.2.3 Preparation of Tris-acetate-EDTA (TAE) buffer

The TAE buffer was prepared by adding 40 ml of the 25 times TAE stock buffer with 960 ml of distilled water to obtain 1000 ml of TAE buffer.

3.2.4 Preparation of Tris-EDTA (TE) buffer

TE buffer was made from 1 M stock of Tris-Cl at pH 7.5 and 500 mM stock of EDTA at pH 8.0. To make 1 litre of TE buffer, 10 ml 1 M Tris-Cl at pH 7.5 was added with 2 ml of 500 mM EDTA at pH 8.0. The mixture was then topped up with 988 ml of distilled water to obtain a total of 1 litre TE buffer.

3.2.5 Preparation of primer working solution

The concentration of the primer stock for both ITS 4 and ITS 5 was 100 µM. The concentration in the Polymerase Chain Reaction (PCR) mixture should be 0.5 µM. Therefore a primer working solution of 25 µM was prepared by adding 12.5 µl of primer stock and 37.5 µl of TE buffer to obtain 50 µl of primer working solution.

3.2.6 Polymerase chain reaction (PCR)

The PCR reaction mixture consisted of 25 µl of PCR mix from QIAGEN, 1 µl of ITS 4 primer, 1 µl of ITS 5 primer, 2 µl of template DNA and 21 µl of RNase free water. The mixture was then placed into the thermal cycler and for initial amplification, PCR reaction mixtures were denatured for 5 minutes at 96°C, followed by 35 PCR cycles for 30 seconds at 95°C, 30 seconds at 52°C, and 90 seconds at 72°C. The final extension step was at 72°C for 10 minutes. This cycle was programmed for 35 reactions.

After the PCR, the resultant products were runned on the agarose gel submerged in TAE buffer solution in a gel electrophoresis tank. The electrophoresis was runned at 100 Volt for 30 minutes. The presence of a distinct thick band indicated a successful PCR run. The PCR products were then sent for PCR clean-up and DNA sequencing to First Base Laboratories Sdn. Bhd for direct sequencing using the same primers.

3.2.7 Alignment of DNA sequences using MEGA 5.0 and Basic Local Alignment Search Tool (BLAST)

The ITS 1 and 2 DNA sequences of endophytic fungi were aligned using the MEGA 5.0 software and the BLAST program at the US National Centre for Biotechnology Information (NCBI), (http://www.ncbi.nlm.gov/) was employed for nucleotide sequence analysis and database searches to identify the putative genus or species of the fungi. The nucleotide sequences of the endophytic fungi strains were aligned against their respective test strains to further confirm their match and thus validating the identification.

3.3 Preliminary screening of marine derived endophytic and manglicolous fungi using the plug assay method

The preliminary screening for antibacterial activity of endophytic and manglicolous fungi was carried out using a mycelial plug assay method as described by Hoskisson *et al.* (2001), Ezra *et al.* (2004) and Zainuddin *et al.* (2008).

Prior to the bioassay, the fungi isolates were cultured on potato dextrose agar (PDA) without antibiotic supplements for one week at 37°C. The test bacteria used comprised of both Gram positive and negative strains. The Gram positive bacteria used were, *Staphylococcus aureus* Rosenbach MTCC 96 (ATCC 9144), *Bacillus subtilis* (Ehrenberg) Cohn. (ATCC 6051), *Bacillus cereus* Frankland and Frankland (ATCC 11778), *Micrococcus luteus* (Schroeter) Cohn (ATCC 49732) and *Enterococcus faecalis* (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC 29212). The Gram negative bacteria used were *Escherichia coli* (Migula) Castellani & Chalmers MTCC 443 (ATCC 25922) and *Pseudomonas aeruginosa* (Schroeter) Migula (ATCC 27853). The test bacteria were provided by the Microbiology Department, University of Malaya and were maintained on Luria Broth (LB) agar at 4°C.

To inoculate the test bacteria, a few colonies were picked using a sterile inoculation loop and transferred to a universal bottle containing Mueller Hinton broth (MHB) and shaken well. A bacterial suspension of each test bacteria was prepared according to the concentration of the 0.5 McFarland standard. The 0.5 McFarland standard was prepared by adding 0.5 ml of 1.174% (wt/vol) barium chloride dehydrate ($BaCl_2 2H_2O$) solution to 99.5 ml of 1% (vol/vol) sulphuric acid. The absorbance of the 0.5 McFarland standard was checked using a spectrophotometer set at a wavelength of 625 nm. A sterile cotton swab was dipped into the adjusted test bacterial suspension and slightly pressed against the inside wall of the tube to remove excess fluid. This cotton

swab was then used to swab the entire surface of the LB agar. The agar plates were divided into four sections and 6 mm agar plugs was cut out from each section of the agar plate to form wells. A 6 mm plug of fungal mycelia was then taken from the edge of an actively growing fungal culture to replace the well in the agar plates inoculated with the test bacteria. The plates were then incubated at 37°C for 24 hours. The diameter of the zone of inhibition was measured in millimetre (mm) and a zone of inhibition of 8 mm in diameter or higher is taken as a positive response (Zainuddin *et al.*, 2008). The experiment was conducted in triplicates. All preparative procedures of media are attached as Appendix 1.

3.4 Determination of minimum inhibitory concentration (MIC) values of marine derived endophytic and manglicolous fungi extracts

The marine derived endophytic and manglicolous fungi strains which demonstrated promising antibacterial activity in the plug assay was further investigation through the broth microdilution assay. This was done to obtain quantitative results from the qualitative results obtained from the plug assay, thus further asserting the antibacterial activity exhibited by these fungi strains.

3.4.1 Extraction of fungal secondary metabolites

The fungi isolates were grown on sterile PDA in Petri plates for 14-30 days at 25°C. Active growing fungi mycelium was then cut into 6 mm plugs and three of those mycelial plugs were added into conical 250 ml flasks containing potato dextrose broth (PDB) and the flasks were incubated at 25°C under shaken phase of 120 rpm for thirty days. After the incubation period, the fungal biomass was separated by filtration. The filtrate was then extracted with equal volumes of ethyl acetate (EA) (1:1). The organic EA layers were then combined and evaporated to dryness using a rotary evaporator at 25°C. The resulting crude obtained was weighed and stored at -20°C prior to use.

3.4.2 Preparation of p-Iodonitrotetrazolium violet (INT) and antibiotics

To prepare INT, 0.4 mg was dissolved in 1 ml of distilled water. As for antibiotics chloramphenicol and penicillin, 2.0 mg of powder was dissolved in 1 ml of distilled water with respect to each antibiotic. Both solutions were stored at -4°C prior to use. Preparative procedures of chemicals are attached as Appendix 1.

3.4.3 Preparation of bacterial suspension

The bacterial suspension was prepared according to the 0.5 McFarland standard. Firstly, three to five isolated colonies were taken from a pure 24-hour bacterial culture at 37°C on the MHA medium with a sterile inoculation loop and inoculated into a sterile Falcon tube containing 3 ml of MHB solution. The suspension was shaken well and the absorbance value was adjusted between 0.08-0.10 at 625 nm wavelength using a spectrophotometer. At this adjusted absorbance value, the bacterial concentration would be 1×10^8 cfu/ml similar to a 0.5 McFarland standard as mentioned in sub-chapter 3.3 above. The bacterial suspension was then diluted to 1×10^6 cfu/ml in order to achieve the final concentration of 5×10^5 cfu/ml after the addition of 50 µl of bacterial suspension into the well.

3.4.4 Preparation of crude stock

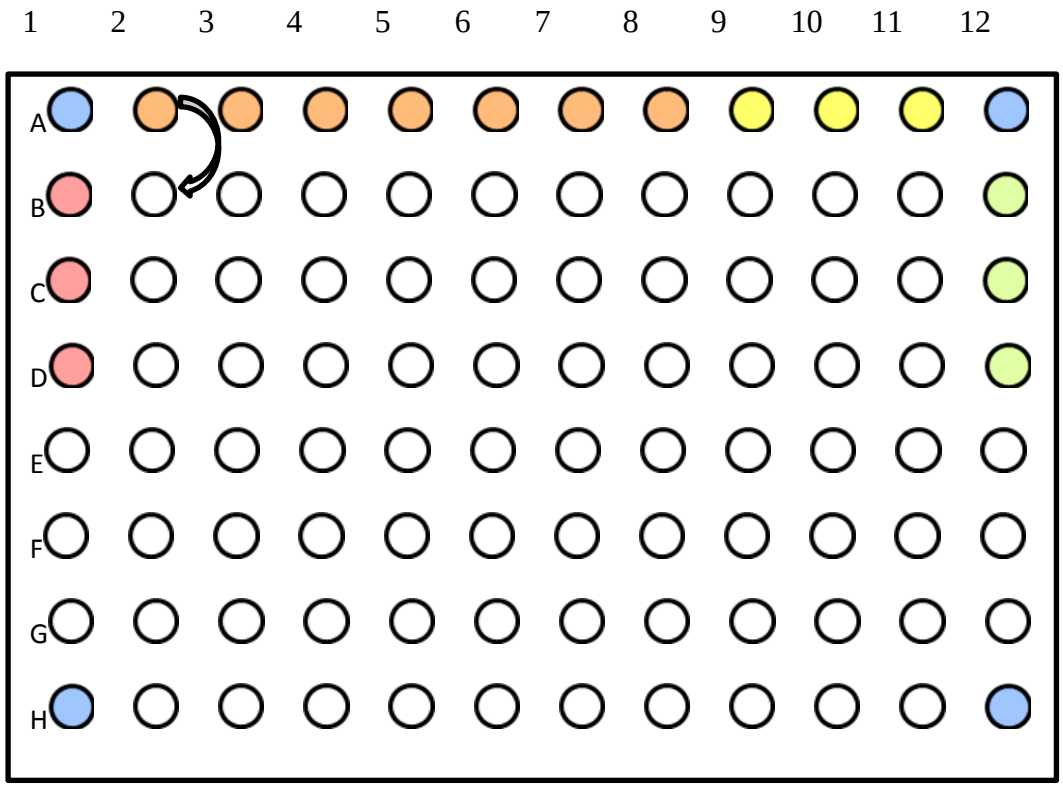
Forty milligrams of each extract was dissolved in 1% Dimethyl sulfoxide (DMSO) to obtain a concentration of 10 mg/ml of stock solution. The solution was sonicated to ensure homogeneity. The solution was then filtered using 0.45 µm syringe filter into a sterile eppendorf tube and kept at -20°C.

3.4.5 Bioassay using broth microdilution method

A sterile 96-well plate was labelled under aseptic conditions as shown in Figure 3.2. Firstly, 100 µl of Mueller-Hinton broth (MHB) was added to the wells at each corner of the plate, labelled blue in Figure 3.2 to serve as sterility control. The wells labelled pink acted as growth control, containing 50 µl MHB and 50 µl bacteria. The wells labelled green acted as negative control, containing 75 µl MHB and 25 µl extract. Fifty millilitres of MHB was added to all the wells in column 2-11, and 50 µl of different fungi extracts in 1% DMSO were pipetted into the first row of the 96 well plate

(Column 2-7). Two-fold serial dilutions were performed using a multichannel micropipette. Wells in columns 2 to 8 contained the same fungi extracts but with dilutions, 2.0, 1.0, 0.50, 0.25, 0.125, 0.063, 0.031 and 0.016 mg/ml while wells in column 9 to 11 contained 50 µl of antibiotic with dilutions, 2.0, 1.0, 0.50, 0.25, 0.125, 0.063, 0.031 and 0.016 mg/ml. Finally 50µl of bacterial suspension (1×10^6 cfu/mL) was added to each tested wells and growth control to achieve a final concentration of 5×10^5 cfu/ml. The 96 well plates were put on the orbital shaker for 10 minutes and then incubated at 37ºC for 24 hours.

After 24 hours, 20 µl of INT were added into each of the wells, and placed on the orbital shaker for 10 minutes, and then incubated at 37ºC for 30 minutes. The changes in colour were then observed. The wells that remained colourless or clear meant that the extract was active against the bacteria while the wells that observed pink colour meant that there were bacteria present and thus indicating that the extract was inactive againt the bacteria. The lowest concentration of the extract at which there was no colour changes in the well was taken as the MIC value. The assay was carried out in triplicates to ensure the accuracy of results.



Sterility control (100 µl MHB)

Growth control (50 µl MHB + 50 µl bacterial suspension)

Negative control (25 µl fungi extracts + 75 µl MHB)

Positive control (50 µl antibiotic + 50 µl bacterial suspension)

50 µl fungi extracts and 50 µl MHB were added to first well then diluted 50µl from first well to second well and so on until the last well, 50 µl of extracts were then discarded, after dilution, 50 µl bacteria suspension was added.

Two-fold serial dilutions

Figure 3.2: Illustration of the 96 well plate used in broth microdilution assay

3.5 Chemical analysis of ethyl acetate crude extract (EAS) of *Saccardoella rhizophorae*

3.5.1 Extraction of crude fungal extract for fractionation

The fungi, *S. rhizophorae* was grown in 3 L of PDB supplemented with 25 ppm of marine salt. The culture was incubated at a temperature of 25°C for 15 days at an agitation rate of 100 rpm. At the end of the incubation, the broth was filtered, separating the fungal mass and broth solution. The broth solution was partitioned with equal volumes of EA and the resulting organic layer was separated. The fungal mass was dried and extracted with EA at a ratio of 1g of dried fungal mass: 10 ml of EA. Both EA extracts from the broth solution and the dried fungal mass was pooled together and evaporated to dryness. The resulting dried EAS of *S. rhizophorae* was then stored at -4°C prior to use.

3.5.2 Solvent sequential extraction

The dried EAS of *S. rhizophorae* was partitioned by sequential extraction using solvents of increasing polarity starting with, *n*-hexane, dichloromethane and methanol. The dried EAS of *S. rhizophorae* was first soaked in *n*-hexane at a ratio of 1 g: 10 ml solvent and shaken in an orbital shaker for 7 hours. The solvent mixture was filtered using Whatman filter paper to separate the solution from undissolved solids and the resulting solution was evaporated to dryness. The remaining undissolved solids on the filter paper were then dried and soaked dichloromethane following the same procedure. This was repeated with the methanol. The last solvent methanol resulted in no undissolved solids. This partitioning procedure afforded a total of three fractions; *n*-hexane (HS), dichloromethane (DS) and methanol (MS) fractions.

3.5.3 Column chromatography

Column chromatography was carried out using silica gel (Merck 230-400 mesh ASTM). The ratio of the silica gel to sample loaded was approximately 30:1. The silica was made into a slurry using dichloromethane before packing into the glass column. The sizes of glass column used in the experiment were of various dimensions as it depended on the weight of crude extract and partially purified fractions. Antibacterial test on fractions HS, DS and MS showed that only two of the three fractions (HS and DS) displayed antibacterial activity. Therefore, bioassay-guided fractionation was carried out on fractions HS and DS. To obtain sub-fraction, HS fraction was dissolved in *n*-hexane and subjected to column chromatography using a gradient solvent system of 100% *n*-hexane, 90% *n*-hexane: 10% EA, 85: 15, 80: 20, 75: 25, 70: 30, 65: 35, 60: 40, 55: 45, 50: 50, 100% EA and 100% acetone. To obtain sub-fractions, DS fraction was dissolved in dichloromethane and was fractionated using a gradient solvent system of 100% dichloromethane, 95% dichloromethane: 5% EA, 90: 10, 85: 15, 80: 20, 75: 25, 70: 30, 65: 35, 60: 40, 55: 45, 50: 50, 100% EA and 100% acetone. Individual column fractions were combined according to their TLC profiles.

3.5.4 Preparative thin layer chromatography (TLC)

Preparative TLC was carried out on Merck 60 F_{254} silica gel plates. Standard chromatograms of sub-fractions were prepared by applying a small drop of the sub-fraction to a silica gel TLC plate and developing it using different solvent systems, to improve separation under saturated conditions. Separated bands were detected by UV-light at 254nm and 365 nm. Upon optimization of the solvent system, the sub-fractions were further purified using the same solvent system. The purity of the compound isolated was confirmed by observing their respective NMR spectra.

3.5.5 Structure elucidation of the isolated secondary metabolite

3.5.5.1 Nuclear magnetic resonance spectroscopy (NMR)

Structures were elucidated mainly using one and two dimensional Nuclear magnetic resonance (NMR) techniques and Liquid chromatography-mass spectrometry (LC/MS). NMR spectra of extracts and pure compounds were recorded using JEOL ECA 400 MHz and JEOL LA 400 MHz NMR spectrometers in chloroform D1 ($CDCl_3$) as a solvent and tetramethysilane as internal standard. The observed chemical shifts (δ) values were recorded in ppm.

3.5.5.2 Liquid chromatography-mass spectroscopy (LCMS)

The Liquid chromatography-mass spectrum-Ion trap-Time of flight (LC-MS-IT-TOF) spectra were recorded on a Ultra-fast liquid chromatography (UFLC) Shimadzu Liquid Chromatograph with a SPD-M20A diode array detector coupled to a IT-TOF The IT-TOF was operated in positive ion electrospray mode.

3.5.5.3 Infra-red spectroscopy

Infrared light is the region of the electromagnetic spectrum with longer wavelength and lower frequency in comparison to visible light. Different molecules absorb specific frequencies of infrared light with respect to their structure thus this spectroscopic technique is used in the identification and study of chemicals. IR spectra were recorded using a Perkin-Elmer Spectrum 400 FT-IR Spectrometer.

3.5.5.4 Ultraviolet-visible spectroscopy

Ultraviolet-visible (UV) light is light in the visible and adjacent near-ultraviolet and near-infrared regions of the electromagnetic spectrum. UV spectroscopy is used in the determination and quantification of chemical based on their different absorption energies. UV-visible spectrum was recorded using a Shimadzu 1650 PC UV-Vis Spectrophotometer.

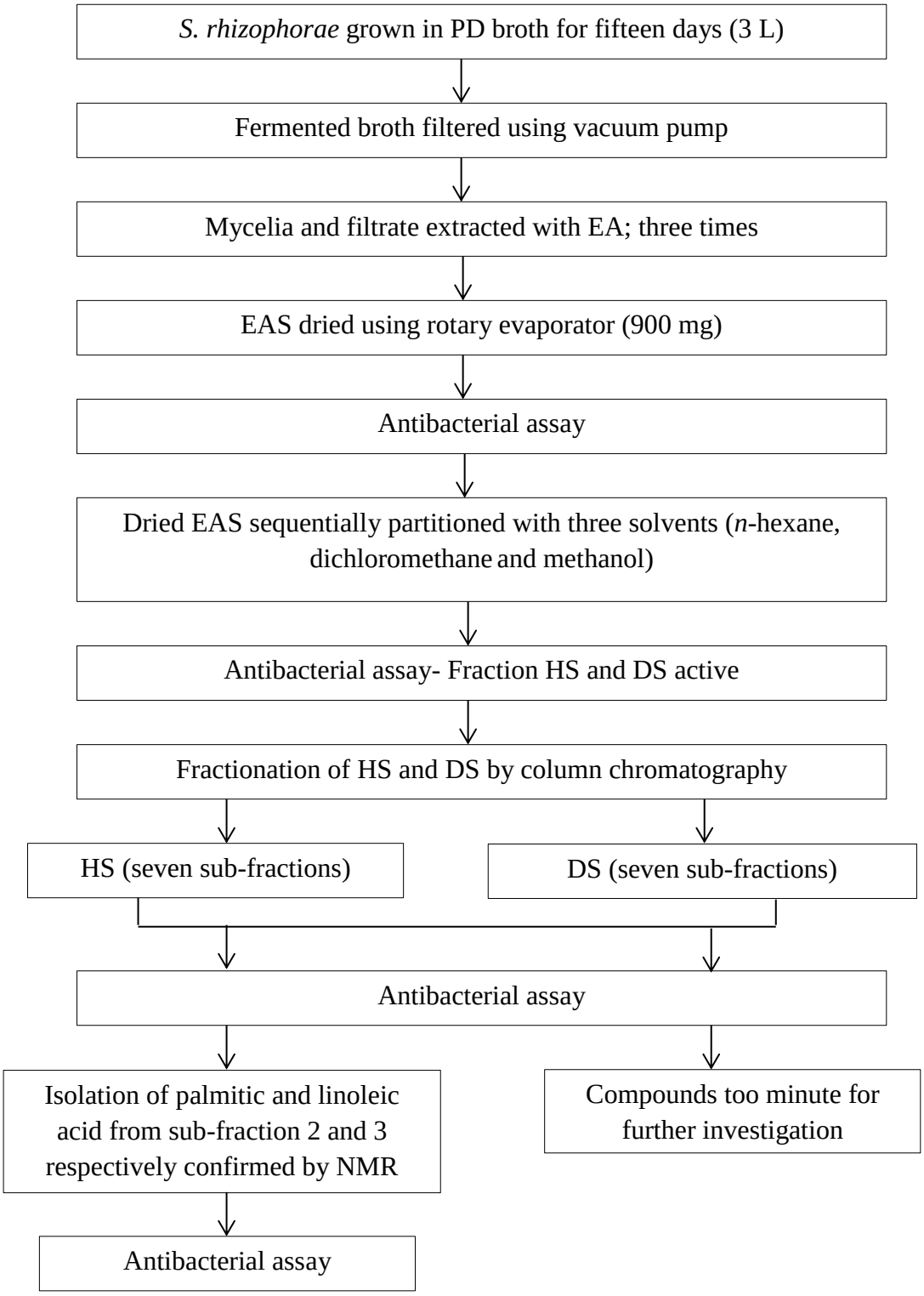


Figure 3.3: Schematic protocol of isolation of the bioactive metabolite by bioassay guided fractionation from *S. rhizophorae*

3.6 Chemicals, media & kits

The chemicals and media used in this study are listed in Table 3.2.

Table 3.2: List of chemicals and media

Chemicals/Media	Manufacturer
Acetone	Systerm Chem AR
Dichloromethane	Systerm Chem AR
Ethyl acetate	Systerm Chem AR
Methanol	Systerm Chem AR
n-hexane	Systerm Chem AR
p-Iodonitrotetrazolium violet (INT)	R&M Marketing, Essex, U.K
Mueller-Hinton agar (MHA)	Oxoid LTD, Basingstoke
Mueller-Hinton broth (MHB)	Oxoid LTD, Basingstoke
Luria-Broth (LB) agar	Merck
Potato Dextrose agar (PDA)	Difco
Potato Dextrose broth (PDB)	Difco
Chloroform-D1	Merck
Silica gel 60	Merck
TLC Silica gel 60	Merck
Agarose	NextGene
TAE Buffer	Bio Basic Inc
Tris (hydroxymethyl) aminomethane hydrochloride	Merck
Ethylenedinitrilotetraacetic acid (EDTA)	Merck
PCR Master mix	QIAGEN
DNeasy® Plant Mini kit	QIAGEN

3.7 Instruments and Labware

The instruments and labware used in this study are listed in Table 3.3.

Table 3.3: List of intruments and lab ware

Instruments/Lab ware	Manufacturer
Autoclave machine	TOMY
Beaker	GQ
Bunsen burner	CAMPINGAZ, Kolon International Corp, Korea
Conical flask	Bomex
Spectrophotometer	Eppendorf
Freezer	Fischer Scientific™
Refrigerator	WELTEX, Refrigeration & Equipment
Laminar flow hood	ESCO
Electronic balance	Sartorius
Rotary evaporator	BUCHI
Schott bottle	DURAN®
Measuring cylinder	NORMAX
Sterile swab	NA
Flat-bottom polysterene 96 well plate	NUNC
Syringe	BD
Parafilm	PECHINEY Plastic Packaging, Chicago, IL
UV Illuminator	
UV Spectrophotometer	Perkin-Elmer Spectrum 400 FT-IR Spectrometer
IR Spectrometer	Shimadzu 1650 PC UV-Vis Spectrophotometer
Orbital shaker	WiseShake®, Wisd
Eppendorf tubes	Axygen® scientific
Membrane filter	Minisart® Sartorius
Micropipette	Transferpette®
Capillary tube	NA
TLC glass chamber	NA
Glass column	NA
Test tube	NA
Glass dropper	NA
Glass vial	NA
NMR tube	Schott Duran
NMR	JEOL (JEOL Ltd., Tokyo, Japan) ECA 400 MHz
LCMS	Shimadzu
HR-ESI-MS	Thermo Instrument (Thermo Fisher Scientific, Bremen, Germany) LTQ XL/LTQ Orbitrap Discovery system
Fume Hood	Cole-Parmer

Table 3.3, continued

Instruments/lab ware	Manufacturer
Round bottom flask	BUCHI
Filter paper	Whatman®, Schleicher & Schuell
Centrifuge	Eppendorf
Vortex	LMS Co, LTD
Electrophoresis gel tank and adapter	CS
Water bath	WiseTherm, Wisd

NA: not applicable

CHAPTER 4

RESULTS

4.1 Endophytic fungi strains from marine associated plants

4.1.1 Endophytic fungi from Vitex rotundifolia and Ipomoea pes-caprae

A total of 34 endophytic fungi were isolated from both marine associated plants including thirteen from *V. rotundifolia* and twenty-one strains from *I. pes-caprae*. The thirteen endophytic fungi strains identified from *V. rotundifolia* comprised of seven genera/species of fungi. Based on Table 4.1, six strains were identified to species level, while six were identified to genus level. The twenty-one strains from *I. pes-caprae*, were comprised of fifteen genera/species of fungi. Alignment of ITS DNA sequences are attached in Appendix 2. As shown in Table 4.1, five strains were identified to its species level while fifteen strains were identified to genus level. The photographs of selected endophytic fungi strains from isolated from *V. rotundifolia* and *I. pes-caprae* are shown in Figure 4.1 and 4.2 respectively. Certain species/genera of endophytic fungi occured in each plant whereby they were of the same species/genus but could be different strains as suggested by their differing GenBank accession numbers as shown in Table 4.1. Overlapping strains isolated from *V. rotundifolia* were *Cochliobolus eragrostidis* (two strains), *Curvularia* sp. (two strains), *Nemania primolutea* (two strains) and *Phoma* sp. (three strains). Overlapping strains from *I. pes-caprae* were *Bipolaris* sp. (four strains), *Dothideomycete* sp. (two strains) and *Phoma* sp. (two strains). Endophytic fungi strains such as *Phoma* sp. and *Guignardia mangiferae* appeared common to the two marine associated plants as they were isolated both from *V. rotundifolia* as well as *I. pes-caprae*.

Table 4.1: Putatively named endophytic fungi from the marine associated plants *V. rotundifolia* and *I. pes-caprae*

Fungi	Plants			
	V. rotundifolia	**GenBank accession**	***I. pes-caprae***	**GenBank accession**
Bipolaris sp.	-		++++	GU017499.1 HQ631009.1 JX960589.1 JX949177.1
Clasdosporium cladosporioides (Fresen.) G.A. de Vries	-		+	EU497957.1
Cochliobolus eragrostidis (Tsuda & Ueyama) Sivan.	++	JN943412.1 JN943448.1	-	
Cochliobolus lunatus R.R. Nelson & F.A. Haasis	-		+	GQ169765.1
Colletotrichum hippeastri Yan L. Yang, Zuo Y. Liu, K.D. Hyde & L. Cai	-		+	JX010293.1
Colletotrichum sp.	-		+	JN390940.1
Curvularia sp.	++	GQ184733.1 HQ631061.1	-	
Dothideomycete sp.	-		++	HQ631008.1 JQ759890.1
Fungal endophyte sp g88	-		+	HM537063.1
Fungal sp. ARIZ B233	+	FJ612966.1	-	
Fusarium equiseti (Corda) Sacc.	-		+	EU595566.1
Guignardia mangiferae A.J. Roy	+	KC686598.1	+	EU273524.1
Letendraea helminthicola (Berk & Broome) Weese	+	JQ026217.1	-	
Minimidochium sp.	-		+	HQ630974.1
Montagnulaceae sp.	-		+	KC291642.1
Myrothecium sp.	-		+	HQ631067.1
Nemania primolutea Y.M. Ju, H.M. Hsieh & J.D. Rogers	++	EF026121.1 KC845930.1	-	
Paecilomyces sp.	+	HQ631041.1	-	
Penicillium sp.	-		+	JQ889696.1
Phoma sp.	+++	HQ630963.1 JN207285.1 JQ388280.1	++	HQ630963.1 JQ388280.1
Pleosporales sp.	-		+	HQ631015.1
Xylaria sp.	-		+	EF423534.1

"-" = represents absence of strain
"+" = represents presence and number of strains

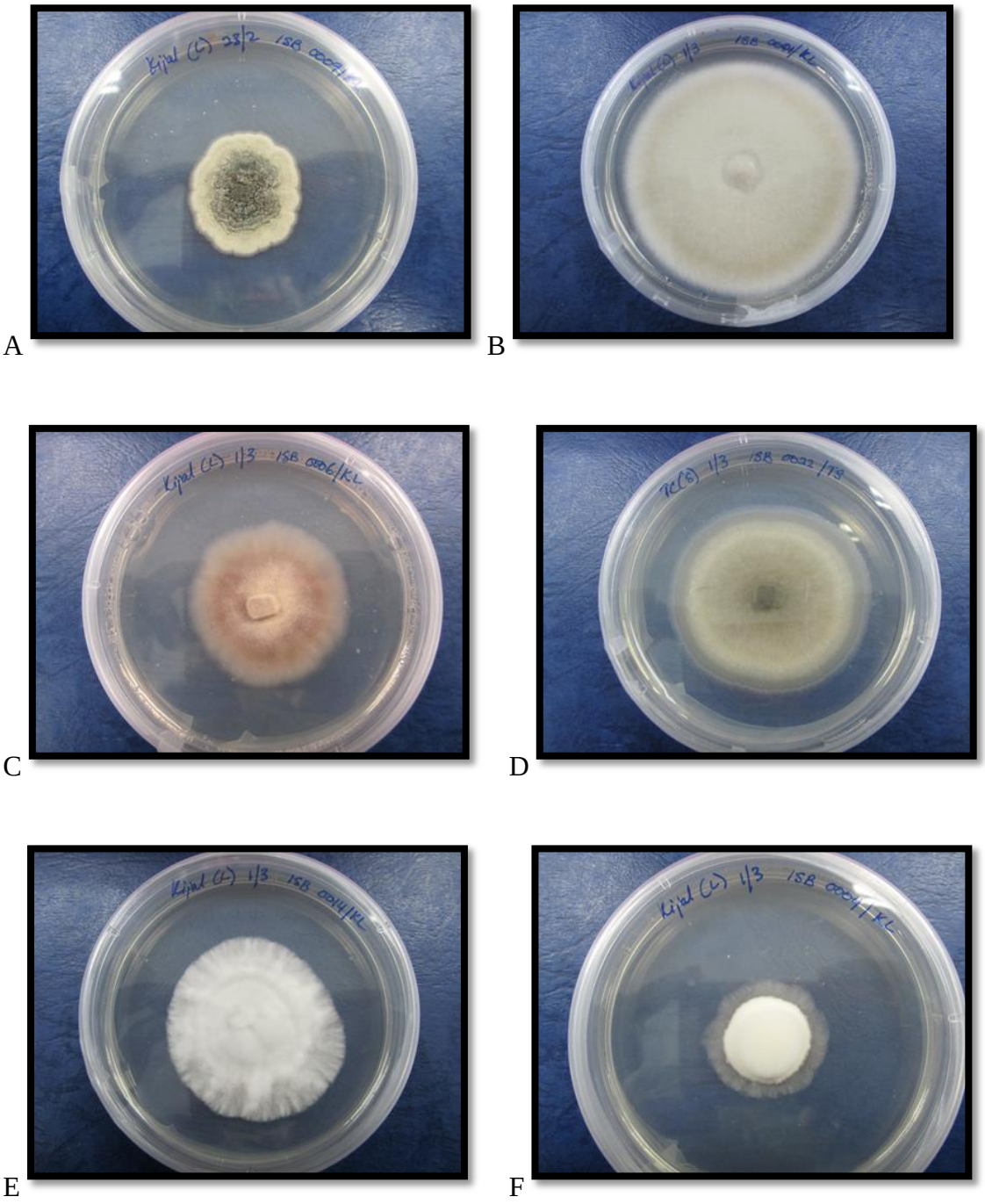


Figure 4.1: Photographs of selected endophytic fungi isolated from *V. rotundifolia* on PDA. A: *G. mangiferae*; B: *Phoma* sp.; C: *L. helminthicola*; D: *C. eragrostidis*; E: *Paecilomyces* sp.; F: Fungal sp. ARIZ B233

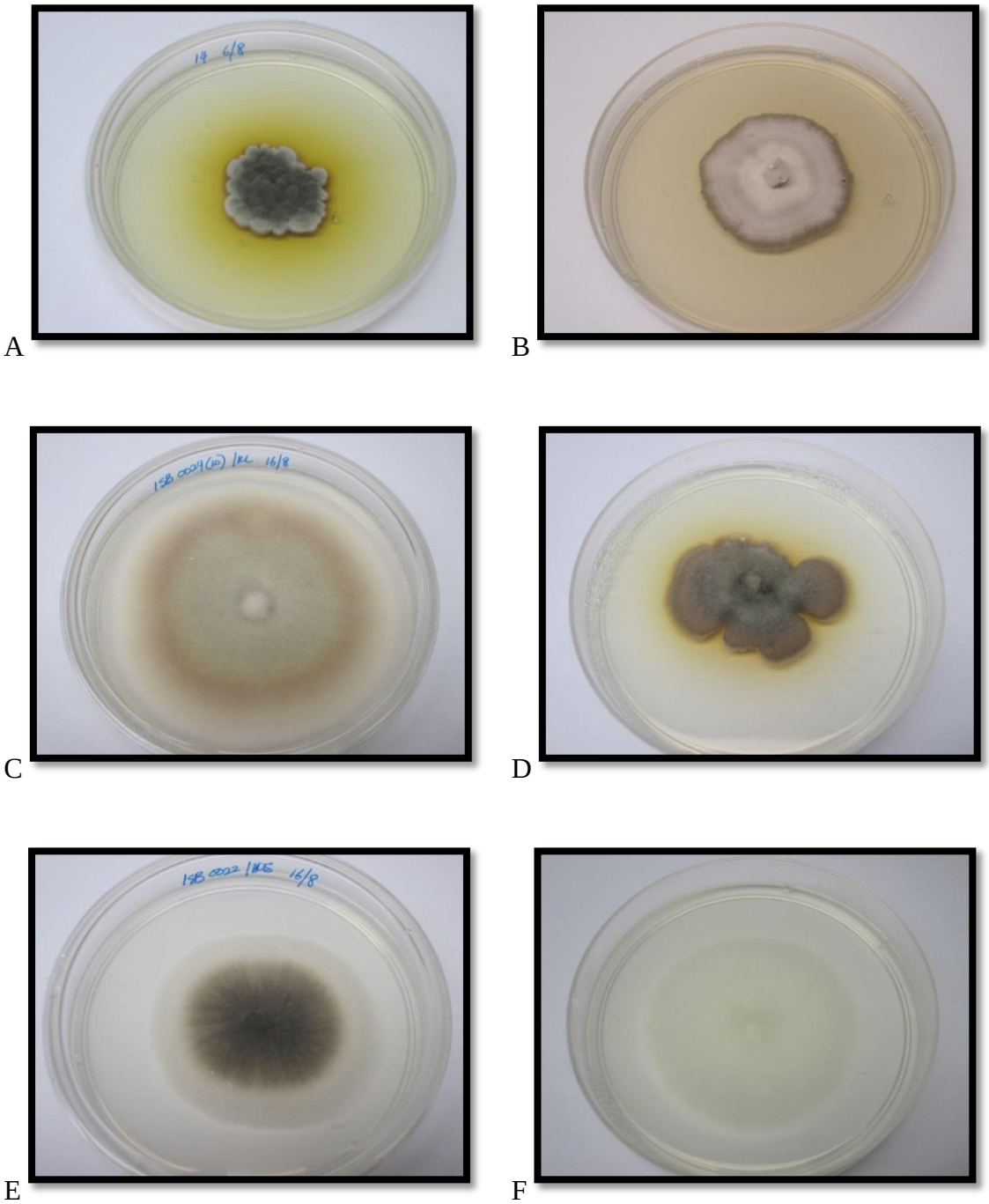


Figure 4.2: Photographs of selected endophytic fungi isolated from *I. pes-caprae* on PDA. A: *C. cladosporioides*; B: *Dothideomycete* sp.; C: *Phoma* sp.; D: *Bipolaris* sp.; E: *F. equiseti*; F: *Colletotrichum* sp.

4.2 Antibacterial activity of marine derived fungi based on plug assay

4.2.1 Antibacterial activity of endophytic fungi from Vitex rotundifolia and Ipomoea pes-caprae

Table 4.2 shows the results obtained from the antibacterial activity screening of the 34 endophytic fungi using the plug assay method. As shown in Table 4.2, five of the thirteen isolated endophytic fungi strains from *V. rotundifolia* displayed antibacterial activity against at least one test bacteria while ten of the twenty-one isolated endophytic fungi strains from *I. pes-caprae* displayed antibacterial activity against at least one test bacteria. Results obtained showed that eight and eleven fungi strains from *V. rotundifolia* and *I. pes-caprae* respectively did not exhibit antibacterial activity against any of the test bacteria. The endophytes from *V. rotundifolia* displayed selective antibacterial activity towards only Gram positive bacteria namely; *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis*. Two fungi strains exhibited antibacterial activity against *S. aureus*, five strains against *B. cereus* and four strains against *B. subtilis*. The fungi strains from *V. rotundifolia* which displayed the broadest spectrum of antibacterial activity which was against three test bacteria were; *Paecilomyces* sp. and *Curvularia* sp.

As for the endophytic fungi strains from *I. pes-caprae*, eight of fungi strains exhibited antibacterial activity against *S. aureus*, seven against *B. cereus*, six against *B. subtilis*, eight against *Micrococcus lutues*, one against *Enterococcus faecalis*, three against *Escherichia coli*, and one against *Pseudomonas aeruginosa*. The broadest spectrum of antibacterial activity was exhibited by *Bipolaris* sp. (HQ631009.1) which was against six out of the seven test bacteria comprising of both Gram positive and negative test bacteria. Although most of the endophytic fungi strains from *I. pes-caprae* displayed antibacterial activity against Gram positive bacteria, some of the fungi strains

were potent against Gram negative bacteria such as; *Dothideomycete* sp. (HQ631008.1) exhibited antibacterial activity against *P. aeruginosa* and fungi strains *Minimidochium* sp., *Bipolaris* sp. (HQ631009.1), and *Penicillium* sp. exhibited antibacterial activity against *E. coli*. A sample of the zones on inhibition demonstrated by the fungi through the plug assay is shown in Figure 4.3. The diameter of zones of inhibition values are attached as Appendix 3.

Table 4.2: Preliminary antibacterial activities of endophytic fungi from the marine associated plant *V. rotundifolia* and *I. pes-caprae* against seven bacterial strains

Fungi	*V. rotundifolia*		*I. pes-caprae*	
	GenBank accession number	**Activity**	**GenBank accession number**	**Activity**
Bipolaris sp.	-	-	GU017499.1	NA
			HQ631009.1	SA(G), BC(G), BS(M), EF(G), ML(G), EC(W)
			JX960589.1	NA
			JX949177.1	BS(M), ML(G)
C. cladosporioides	-	-	EU497957.1	NA
C. eragrostidis	JN943412.1	NA	-	-
	JN943448.1	BC(G), BS(G)		
C. lunatus	-	-	GQ169765.1	NA
C. hippeastri	-	-	JX010293.1	NA
Colletotrichum sp.	-	-	JN390940.1	NA
Curvularia sp.	GQ184733.1	SA(G), BC(G), BS(G)	-	-
	HQ631061.1	NA		
Dothideomycete sp.	-	-	HQ631008.1	SA(M), BC(M), ML(M), PA(W)
			JQ759890.1	NA
Fungal endophyte sp g88	-	-	HM537063.1	NA
Fungal sp. ARIZ	FJ612966.1	NA	-	-
F. equiseti	-	-	EU595566.1	SA(G), BC(G), BS(G), ML(G)
G. mangiferae	KC686598.1	BC(G), BS(G)	EU273524.1	NA

Table 4.2, continued

L. helminthicola	JQ026217.1	NA	-	-
Minimidochium sp.	-	-	HQ630974.1	SA(W), BC(W), BS(W), ML(M), EC(W)
Montagnulaceae sp.	-	-	KC291642.1	SA(G), BC(G), BS(G), ML(G)
Myrothecium sp.	-	-	HQ631067.1	SA(W), BC(W), BS(W)
N. primolutea	EF026121.1	NA	-	-
	KC845930.1	NA		
Paecilomyces sp.	HQ631041.1	SA(G), BC(G), BS(G)	-	-
Penicillium sp.	-	-	JQ889696.1	SA(W), BC(W), EC(W)
Phoma sp.	HQ630963.1	NA	HQ630963.1	ML(W)
	JN207285.1	BC(W)	JQ388280.1	SA(M), ML(M)
	JQ388280.1	NA		
Pleosporales sp.	-	-	HQ631015.1	NA
Xylaria sp.	-	-	EF423534.1	NA

NA: No activity towards any test bacteria
SA: *Staphylococcus aureus*; BC: *Bacillus cereus*; BS: *Bacillus subtilis*; EF: *Enterococcus faecalis*; ML: *Micrococcus luteus*; EC: *Escherichia coli*; PA: *Pseudomonas aeruginosa*
(W): Weak activity; (M): Moderate activity; (G): Good activity
Activities were classified as: 8 mm to 9 mm: weak activity; 10 mm to 11 mm: moderate activity; > 11 mm: good activity
The diameter of plug well: 6 mm
Experiment carried out in triplicates

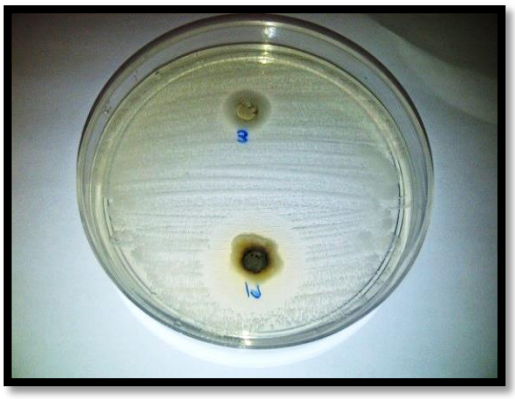
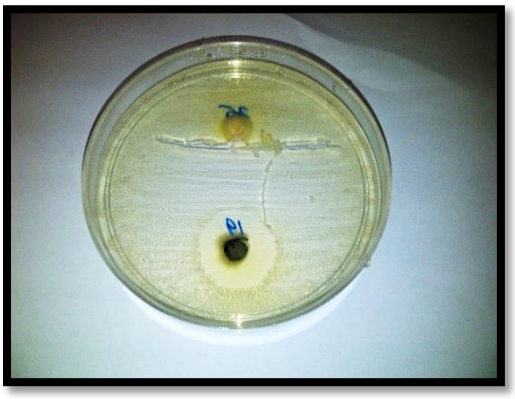
A  B 

Figure 4.3: Photographs of inhibition zone in plug assay. A: Zone inhibition of antibacterial activities of *Bipolaris* sp. and *Montagnulaceae* sp. B: Zone inhibition of antibacterial activities of *Phoma* sp. and *Paecilomyces* sp.

4.2.2 Antibacterial activity of manglicolous fungi

Table 4.3 shows the preliminary antibacterial activities exhibited by the manglicolous fungi in the present study when assessed through the plug assay method. Out of the total eleven fungi strains, ten displayed antibacterial activity against at least one test bacteria. Only *Fusarium* sp. did not exhibit antibacterial activity towards any of the test bacteria. Certain manglicolous fungi used in the present investigation belonged to the same species but are different strains because they were isolated frm different locations, such as the fungi strains; *Dactylospora haliotrepha* (4 strains) and *Verruculina enalia* (two strains) fungi strains. *Staphylococcus aureus* was the test bacteria most susceptible to the antibacterial potential of the manglicolous fungi with nine out of eleven manglicolous fungi exhibiting antibacterial activities against this bacterium. Most of the antibacterial activity displayed by the manglicolous fungi in this study was skewed towards Gram positive bacteria with only one manglicolous fungi, *S. rhizophorae* active against the Gram negative *E. coli*. The test bacteria most susceptible to the antibacterial potential of *S. rhizophorae* displayed promising potential with antibacterial activity against five test bacteria.

Table 4.3: Preliminary antibacterial activity of manglicolous fungi against seven test bacteria

Fungi	Bacterial strains tested						
	S. aureus	***B. cereus***	***B. subtilis***	***M. luteus***	***E. faecalis***	***E. coli***	***P. aeruginosa***
Corollospora maritima	NA	M	M	NA	NA	NA	NA
D. haliotrepha (ISB001)	M	NA	M	NA	NA	NA	NA
D. haliotrepha (ISB002)	M	NA	M	NA	NA	NA	NA
D. haliotrepha (ISB003)	G	G	G	M	NA	NA	NA
D. haliotrepha (ISB004)	G	G	G	NA	NA	NA	NA
Fusarium sp.	NA	NA	NA	NA	NA	NA	NA
Henningsomyces sp.	G	G	G	M	NA	NA	NA
MF 28	G	G	G	NA	NA	NA	NA
S. rhizophorae	G	G	G	G	NA	W	NA
V. enalia (ISB005)	M	G	G	NA	NA	NA	NA
V. enalia (ISB006)	M	G	M	NA	NA	NA	NA

NA: No activity; (W): Weak activity; (M): Moderate activity; (G): Good activity
Activities were classified as: 8 mm to 9 mm: weak activity; 10 mm to 11 mm: moderate activity; > 11 mm: good activity
The diameter of plug well: 6 mm
Experiment carried out in triplicates

4.3 Minimum inhibitory concentration (MIC) values of marine derived fungi crude extracts obtained through broth microdilution assay

4.3.1 MIC values of endophytic fungi crude extracts

The five most promising endophytic fungi strains; *Minimidochium* sp., *Paecilomyces* sp., *Curvularia* sp. (GQ184733.1), *Montagnulaceae* sp. (KC291642.1) and *Bipolaris* sp. (HQ631009.1) were selected for further antibacterial assessment through the broth microdilution assay. As shown in Table 4.4, most of the fungi extracts inhibited the growth of Gram positive bacteria. None of the five fungi extracts were potent against *P. aeruginosa* while *B. cereus* was the only bacterial strain susceptible to the antibacterial activities of all of the five extracts. The extract of *Paecilomyces* sp. showed promising antibacterial potential with low MIC values of 0.250 mg/ml and below against five of the test bacteria. On the other hand, the extract of *Bipolaris* sp. was the only one to exhibit good antibacterial activity against *E. coli* with a low MIC of at 0.250 mg/ml in comparison to *Montagnulaceae* sp.

Table 4.4: Minimum inhibitory concentration (MIC) values of endophytic fungi crude extracts in mg/ml

Endophytic Fungi	Bacteria						
	S. aureus	*B. cereus*	*B. subtilis*	*E. faecalis*	*M. luteus*	*P. aeruginosa*	*E. coli*
Minimidochium sp.	2.000±0	1.000±0	1.000±0	2.000±0	1.000±0	NA	NA
Paecilomyces sp.	0.125±0	0.063±0	0.125±0	0.250±0	0.250±0	NA	NA
Curvularia sp. (GQ184733.1)	NA	2.000±0	1.000±0	NA	NA	NA	NA
Montagnulaceae sp. (KC291642.1)	2.000±0	0.500±0	NA	NA	1.000±0	NA	1.000±0
Bipolaris sp. (HQ631009.1)	0.500±0	0.250±0	NA	NA	0.500±0	NA	0.250±0

Mean ± standard deviation, n=3; NA: no activity

4.3.2 MIC values of manglicolous fungi crude extracts

The MIC values of manglicolous fungi crude extracts were determined using the broth microdilution assay method (Figure 4.4) and results obtained are shown in Table 4.5. All the ten selected manglicolous fungi displayed antibacterial activity against at least one test bacteria. From the results, most of the fungi displayed antibacterial activity against Gram positive bacteria. The fungi strains potent against Gram negative bacteria were *S. rhizophorae* and the unidentified fungi strain, MF28 which displayed activity against *E. coli*.

There were four strains of *D. haliotrepha* that were investigated in this study. According to the results obtained, the fungi strains ISB001 and ISB002 possessed similar activities whereby both exhibited antibacterial activity against the bacteria *S. aureus* and *B. cereus*. Among all four strains, the *D. haliotrepha* strain ISB003 exhibited the widest range of antibacterial activity which was against four of the seven test bacteria namely; *S. aureus*, *B. cereus*, *B. subtilis* and *M. luteus*. The *D. haliotrepha* strain ISB004 displayed an MIC value of 1.0 mg/ml against *S. aureus*, *B. cereus* and *Bacillus subtilis*. There were two strains of the fungi *V. enalia* that were investigated in the present study. Both fungi strains displayed similar antibacterial activities, being effective against *S. aureus, B. cereus* and *B. subtilis* at an MIC value of 1.0 mg/ml.

The fungi strain *S. rhizphorae* was chosen for further investigation and chemical analysis because it was the only manglicolous fungi that exhibited the broadest spectrum of antibacterial activity against five out of the seven test bacteria comprising of four Gram positive bacteria; *S. aureus*, *B. cereus*, *B. subtilis*, *M. luteus* and 1 Gram negative bacteria namely *E. coli*. In addition, the crude extract (EAS) of *S. rhizphorae* exhibited MIC values of 0.500 mg/ml and lower against test bacteria inhibited by its actions. Although crude extracts from fungi such as, *Henningsomyces* sp. and *D.*

haliotrepha (ISB003) also exhibited low MIC values, proving to possess promising potential, none of these fungal crude extracts were active against Gram negative bacteria, which is why *S. rhizophorae* was chosen for further analysis. Although the crude extract of MF 28 also exhibited antibacterial activity against *E. coli*, its MIC value was higher (less potent) in comparison to the MIC value of the crude extract of *S. rhizophorae*.

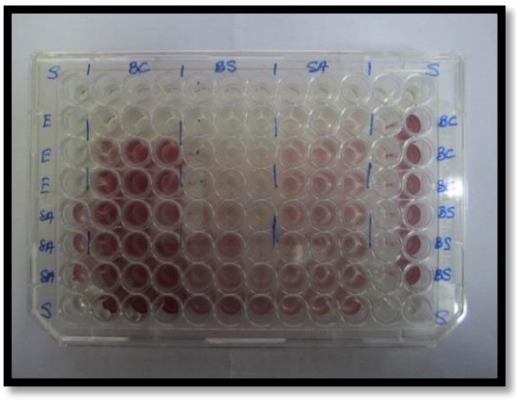
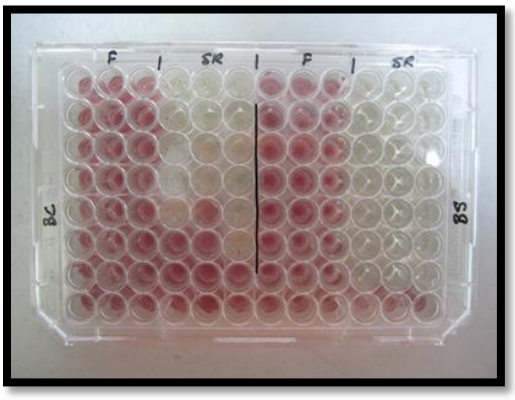
A  B 

Figure 4.4: Photographs of colour changes indicating MIC values in broth microdilution assay. A: MIC of MF28 extract, B: MIC of *S. rhizophorae* extract

Table 4.5: Minimum inhibitory concentration (MIC) values of manglicolous fungi crude extracts in mg/ml

Manglicolous Fungi	**Bacteria**						
	S. aureus	***B. cereus***	***B. subtilis***	***E. faecalis***	***M. luteus***	***P. aeruginosa***	***E. coli***
S. rhizophorae	0.5000±0	0.2500±0	0.0521±0.02	NA	0.5000±0	NA	0.5000±0
MF 28	1.0000±0	1.0000±0	0.2500±0	NA	NA	NA	2.0000±0
Henningsomyces sp.	0.2500±0	1.0000±0	0.4167±0.15	NA	1.0000±0	NA	NA
C. maritima	NA	0.2500±0	0.2500±0	NA	NA	NA	NA
D. haliotrepha (ISB001)	0.5000±0	NA	1.0000±0	NA	NA	NA	NA
D. haliotrepha (ISB002)	0.5000±0	NA	1.0000±0	NA	NA	NA	NA
D. haliotrepha (ISB003)	0.1250±0	0.1250±0	0.0625±0	NA	0.5000±0	NA	NA
D. haliotrepha (ISB004)	1.0000±0	1.0000±0	1.0000±0	NA	NA	NA	NA
V. enalia (ISB005)	1.0000±0	1.0000±0	1.0000±0	NA	NA	NA	NA
V. enalia (ISB006)	1.0000±0	1.0000±0	1.0000±0	NA	NA	NA	NA

Mean ± standard deviation, n=3; NA: no activity

4.4 Sub-fractions from ethyl acetate crude extract (EAS) of *Saccardoella rhizphorae* obtained through bioassay guided fractionation

4.4.1 EAS of Saccardoella rhizophorae

A total weight of 900 mg of crude EA extract (EAS) were obtained from the larger extraction scale of, 3 L of *S. rhizophorae* (Figure 4.5) fermentation broth partitioned with equal volumes of EA. The dried EAS obtained was dark brownish in colouration.

Figure 4.5: Photograph of 15 day old *S. rhizophorae* cultured on PDA media

4.4.2 Fractions of EAS

Three fractions were obtained through solvent partitioning using three solvents in order of increasing polarity from EAS of *S. rhizophorae*, affording fractions, HS, DS, and MS. The weight and colour of each fraction are shown in Table 4.6. The fraction with the highest weight was the DS fraction with a total weight of 320 mg while the other two fractions had lower weight in comparison with 235 mg and 110 mg from the MS and HS fractions respectively.

Table 4.6: Colour and dry weight of HS, DS and MS fractions

Fungi	Fraction	Colour of fraction	Dry weight of fraction (mg)
Saccardoella rhizophorae	HS	Yellow	110
	DS	Dark brown	320
	MS	Brownish black	235

4.4.3 Column chromatography fractionation of HS and DS fractions

4.4.3.1 Sub-fractions of HS fraction

Column fractionation of the HS fraction obtained from the solvent partitioning afforded 43 fractions. The 43 fractions obtained were further pooled into seven sub-fractions based on the similarity of the TLC profiles for each fraction. The weight and appearance of the sub-fractions obtained are shown in Table 4.7.1. Sub-fractions 2, 3 and 4 had significantly higher weights at, 51.1 mg, 24.5 mg and 20.3 mg respectively in comparison to the other 4 sub-fractions. Sub-fraction 1 had the lowest weight at 5 mg.

Table 4.7.1: Weight and appearance of sub-fractions from HS fraction

Sub-fraction	Weight (mg)	Appearance
1	5	Oily, light greenish yellow
2	51.1	Oily, light orange brown
3	24.5	Oily, light greenish
4	20.3	Oily, light yellow
5	5.5	Oily, almost colorless
6	5.6	Oily, very light yellow
7	7.9	Oily, medium brown

4.4.3.2 Sub-fractions of DS fraction

Column fractionation of the DS fraction obtained from the solvent partitioning, afforded 70 fractions in total. The 70 fractions obtained were further pooled into seven sub-fractions based on the similarity of the TLC profiles for each fraction. The weight and appearance of the sub-fractions obtained are displayed in Table 4.7.2. Sub-fractions 4, 5 and 7 had the highest weight at 92.1 mg, 76.2 mg and 47.2 mg respectively. The other sub-fractions had considerably lower weight in comparison.

Table 4.7.2: Weight and appearance of sub-fractions from DS fraction

Sub-fraction	Weight (mg)	Appearance
1	20.1	Light yellowish
2	10.9	Light brownish
3	12.1	Light brownish
4	92.1	Dark brown
5	76.2	Dark brown
6	10.2	Blackish
7	47.2	Blackish

4.5 Antibacterial activity of *Saccardoella rhizophorae* extracts

4.5.1 MIC values of solvent partition fractions

The MIC values of the three fractions obtained from the solvent partitioning extraction are shown in Table 4.8. Out of the three fractions, the HS fraction exhibited the most promising antibacterial activity against six out of seven test bacteria, comprising of both Gram positive and negative namely; *S. aureus*, *Bacillus cereus*, *B. subtilis*, *E. faecalis*, *M. luteus* and *E. coli*. The DS fraction exhibited antibacterial activity against five out of the seven test bacteria all of which comprised of Gram positive bacteria. The MS fraction displayed no antibacterial activity towards any of the test bacteria in this study. From the results obtained, the DS fraction had lower MIC values towards the bacteria against which they exhibited antibacterial activity whereby all MIC values recorded were 0.125 mg/ml and below.

Table 4.8: Minimum inhibitory concentration (MIC) values of HS, DS and MS fractions in mg/ml

Bacteria	**Fractions**		
	HS	**DS**	**MS**
S. aureus	2.0000±0	0.1250±0	NA
B. cereus	0.2500±0	0.0313±0	NA
B. subtilis	0.1250±0	0.0156±0	NA
E. faecalis	1.0000±0	0.1250±0	NA
M. lutues	0.5000±0	0.0625±0	NA
P. aeruginosa	NA	NA	NA
E. coli	2.0000±0	NA	NA

Mean ± standard deviation, n=3; NA: no activity

4.5.2 MIC values of the sub-fractions from the HS fraction

The MIC values of the sub-fractions from the HS fraction are shown in Table 4.8.1. The sub-fractions that displayed antibacterial activity towards the most number of bacterial strains were sub-fractions 2, 3 and 7, all of which exhibited antibacterial activity against

at least three of the seven test bacteria. Sub-fraction 2 exhibited antibacterial activity against *B. cereus, E. faecalis* and *M. luteus*. Sub-fraction 3 exhibited antibacterial activity against *S. aureus, B. cereus* and *B. subtilis* while sub-fraction 7 exhibited antibacterial activity against *S. aureus, B. cereus* and *E. faecalis*. The other 4 sub-fractions exhibited activity against two or less test bacteria with sub-fraction 1, 4 and 6 effective against only one test bacteria. Sub-fraction 1 exhibited antibacterial activity against *E. faecalis*, while sub-fractions 4 and 6 exhibited antibacterial activity against *B. cereus*. The bacterium most susceptible to the action of the sub-fractions from HS fraction was *B. cereus* whereby, all the sub-fractions except sub-fraction 1 were active against this bacterium. Sub-fractions 1, 2 and 7 were active against *E. faecalis* while sub-fractions 3 and 7 were active against *S. aureus* and sub-fractions 3 and 5 displayed activity against *B.subtilis*. Only sub-fraction 2 exhibited antibacterial activity against *M. luteus*. None of the 7 sub-fractions possessed antibacterial activity against the Gram negative test bacteria namely, *P. aeruginosa* and *E. coli*. Although sub-fraction 7 exhibited the lowest MIC values in comparison to the remaining six sub-fractions, the constituents in sub-fraction 7 could not be further elucidated due its low quantity as previously stated in Table 4.7.1. All the MIC values recorded by the sub-fractions of HS fraction were 0.5 mg/ml and below.

4.5.3 MIC values of the sub-fractions from the DS fraction

The MIC values of the sub-fractions of the DS fraction are shown in Table 4.8.2. The sub-fractions from the DS fraction exhibited antibacterial activity only towards the Gram positive test bacteria with no activity exhibited against *E.coli* and *P. aeruginosa* including the Gram positive *E. faecalis*. Out of the seven sub-fractions obtained from further fractionation of the DS fraction, sub-fractions 4 and 5 displayed antibacterial activity against the most number of test bacteria whereby these two sub-fractions were

active against *S. aureus*, *B. cereus*, *B. subtilis* and *M. luteus*. Based on results obtained, the bacterium *M. luteus* was the most susceptible towards the actions of the sub-fractions of DS fraction whereby five out of the seven sub-fractions were active against this bacterium. However due to minute amounts of the sub-fractions of DS fraction no further chemical analysis was possible. This may be undertaken in future studies.

Table 4.8.1: Minimum inhibitory concentration (MIC) values of sub-fractions of HS fraction in mg/ml

Sub-fraction	Bacteria						
	S. aureus	***B. cereus***	***B. subtilis***	***E. faecalis***	***M. luteus***	***P. aeruginosa***	***E. coli***
1	NA	NA	NA	0.5000±0	NA	NA	NA
2	NA	0.2500±0	NA	0.2500±0	0.5000±0	NA	NA
3	0.5000±0	0.2500±0	0.2500±0	NA	NA	NA	NA
4	NA	0.5000±0	NA	NA	NA	NA	NA
5	NA	0.5000±0	0.5000±0	NA	NA	NA	NA
6	NA	0.5000±0	NA	NA	NA	NA	NA
7	0.2500±0	0.0313±0	NA	0.0625±0	NA	NA	NA

Mean ± standard deviation, n=3; NA: no activity

Table 4.8.2: Minimum inhibitory concentration (MIC) values of sub-fractions of DS fraction in mg/ml

Sub-fraction	Bacteria						
	S. aureus	***B. cereus***	***B. subtilis***	***E. faecalis***	***M. luteus***	***P. aeruginosa***	***E. coli***
1	NA	NA	NA	NA	0.2500±0	NA	NA
2	NA	NA	NA	NA	0.5000±0	NA	NA
3	NA	NA	NA	NA	NA	NA	NA
4	0.5000±0	0.5000±0	0.1250±0	NA	0.5000±0	NA	NA
5	0.2500±0	0.2500±0	0.0625±0	NA	0.2500±0	NA	NA
6	NA	NA	NA	NA	0.2500±0	NA	NA
7	NA	NA	NA	NA	NA	NA	NA

Mean ± standard deviation, n=3; NA: no activity

4.5.4 MIC values of metabolites from Saccardoella rhizophorae

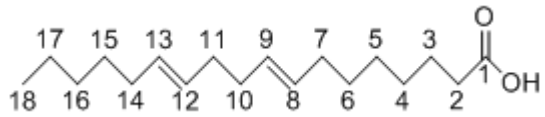
Sub-fractions 2 and 3 from the HS fraction were chosen for further chemical analysis based on their promising antibacterial potential against the most number of test bacteria as well as their low MIC values of not more than 0.500 mg/ml. Linoleic acid was isolated from sub-fraction 2 while palmitic acid was isolated from sub-fraction 3 of the HS fraction. The MIC values of the metabolites from *S. rhizophorae*; linoleic acid and palmitic acid are shown in Table 4.9. Comparing the MIC values of the linoleic acid in relation to the pool from which it was isolated from, the sub-fraction 2 of the HS fraction was active against *B. cereus*, *E. faecalis* and *M. luteus* however, linoleic acid exhibited antibacterial activity towards *S. aureus* and *B. subtilis*. As for palmitic acid which was isolated from sub-fraction 3 of the HS fraction, the pure compound palmitic acid displayed similar antibacterial activity with the sub-fraction from which it was isolated from except the additional exhibition of antibacterial activity against *M. luteus* which was not in sync with its sub-fraction. Both linoleic and palmitic acid, exhibited selected antibacterial activity directed towards only the Gram positive test bacteria. Overall, both linoleic and palmitic acid did not exhibit excellent antibacterial against the test bacteria activity in comparison to the standard antibiotics used in this study, with the lowest MIC at 0.250 mg/ml and highest at 1.000 mg/ml.

Table 4.9: Minimum inhibitory concentration (MIC) of pure compounds in mg/ml

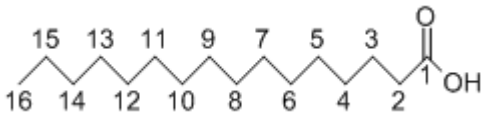
Metabolites	Bacteria						
	S. aureus	*B. cereus*	*B. subtilis*	*E. faecalis*	*M. luteus*	*P. aeruginosa*	*E. coli*
Palmitic acid	0.500±0	0.250±0	0.250±0	NA	0.500±0	NA	NA
Linoleic acid	1.000±0	NA	0.500±0	NA	NA	NA	NA
Penicillin	0.004±0	0.063±0	0.063±0	0.500±0	0.125±0	0.500±0	0.125±0
Chloramphenicol	0.031±0	0.016±0	0.016±0	0.500±0	0.016±0	0.125±0	0.008±0

Mean ± standard deviation, n=3; NA: no activity

4.6 NMR spectroscopic data of metabolites from *Saccardoella rhizophorae*



Linoleic acid



Palmitic acid

Figure 4.6: Structure of linoleic acid and palmitic acid

Linoleic acid, also known as *cis*-9, *cis*-12-octadecadenoic acid was isolated from sub-fraction 2 of the HS fraction while, palmitic acid, or *n*-hexadecanoic acid was isolated from sub-fraction 3 of the HS fraction. Palmitic acid was isolated as light yellowish powder while linoleic acid was isolated as light yellowish oil. Linoleic acid is a known polyunsaturated fatty acid while palmitic acid is a known saturated fatty acid. Figures 3.6 shows the structure of linoleic acid and palmitic acid after determination using ^{1}H and ^{13}NMR spectra that were recorded in deuterated chloroform ($CDCl_3$) using FT-NMR Lambda 400 MHz and mass spectrum on HP 6890 Series Mass Selective Detector. The moleculer formula of linoleic acid and palmitic acid was obtained as $C_{18}H_{32}O_2$ and $C_{16}H_{32}O_2$ respectively. The ^{1}H and 13C NMR 400Hz data for linoleic acid are tabulated in Table 4.10 while Table 4.11 shows the ^{1}H and 13C NMR 400Hz data for palmitic acid. The ^{1}H NMR and 13C NMR spectrums for linoleic acid are displayed in Figure 4.7a and 3.7b respectively. The 2-dimensional NMR spectrums for linoleic acid are attached as Appendix 4. As for palmitic acid, the ^{1}H and 13C NMR spectrums are displayed in Figure 4.8a and 3.8b respectively. The 2-dimensional NMR spectrums for palmitic acid are attached as Appendix 5.

Table 4.10: The ^{1}H and 13C NMR 400Hz data of linoleic acid

Position	δH[a]	δC
1		180.7
2	2.35 *t* (7.8)	34.3
3	1.64 *m*	24.8
4	1.31-1.38 *m*[b]	29.2[e]
5	1.31-1.38 *m*[b]	29.3[e]
6	1.31-1.38 *m*[b]	29.3[e]
7	1.31-1.38 *m*[b]	29.5[e]
8	2.04-2.09 *m*[b]	27.4[d]
9	5.30-5.42 *m*[b]	130.2[f]
10	5.30-5.42 *m*[b]	128.1[g]
11	2.78 *t* (6.0)	25.8
12	5.30-5.42 *m*[b]	128.3[g]
13	5.30-5.42 *m*[b]	130.4[f]
14	2.04-2.09 *m*[b]	27.3[d]
15	1.31-1.38 *m*[b]	29.8[e]
16	1.31-1.38 *m*[b]	22.8[c]
17	1.31-1.38 *m*[b]	31.7[c]
18	0.90 *t* (7.3)	14.1

[a]Coupling constants (J) in Hz are indicated in parentheses
[b]Overlapping signals
[c,d,e,f,g]Chemical shifts are interchangeable

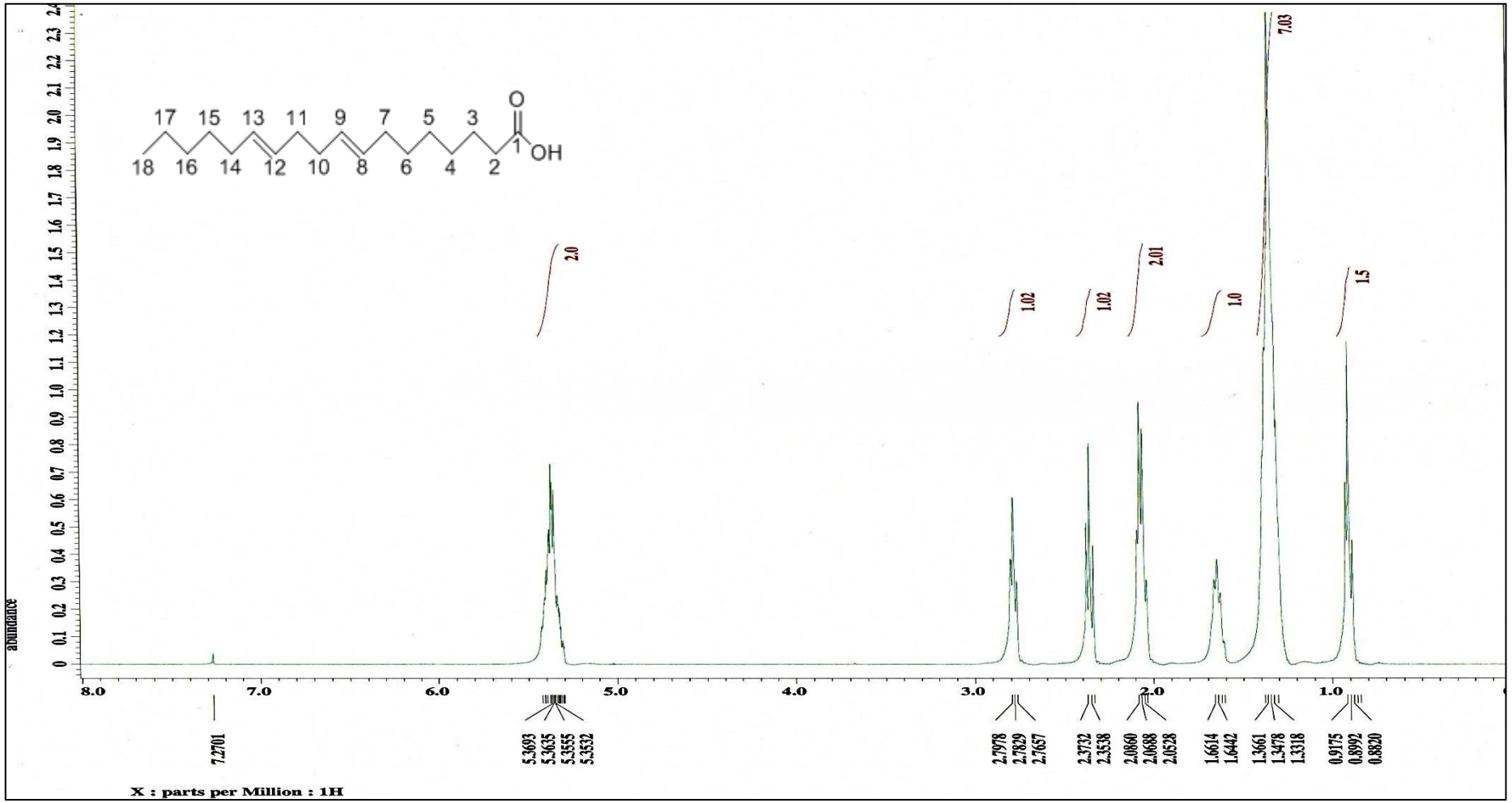


Figure 4.7a: The ^{1}H NMR 400 Hz of linoleic acid in $CDCl_3$ (ppm)

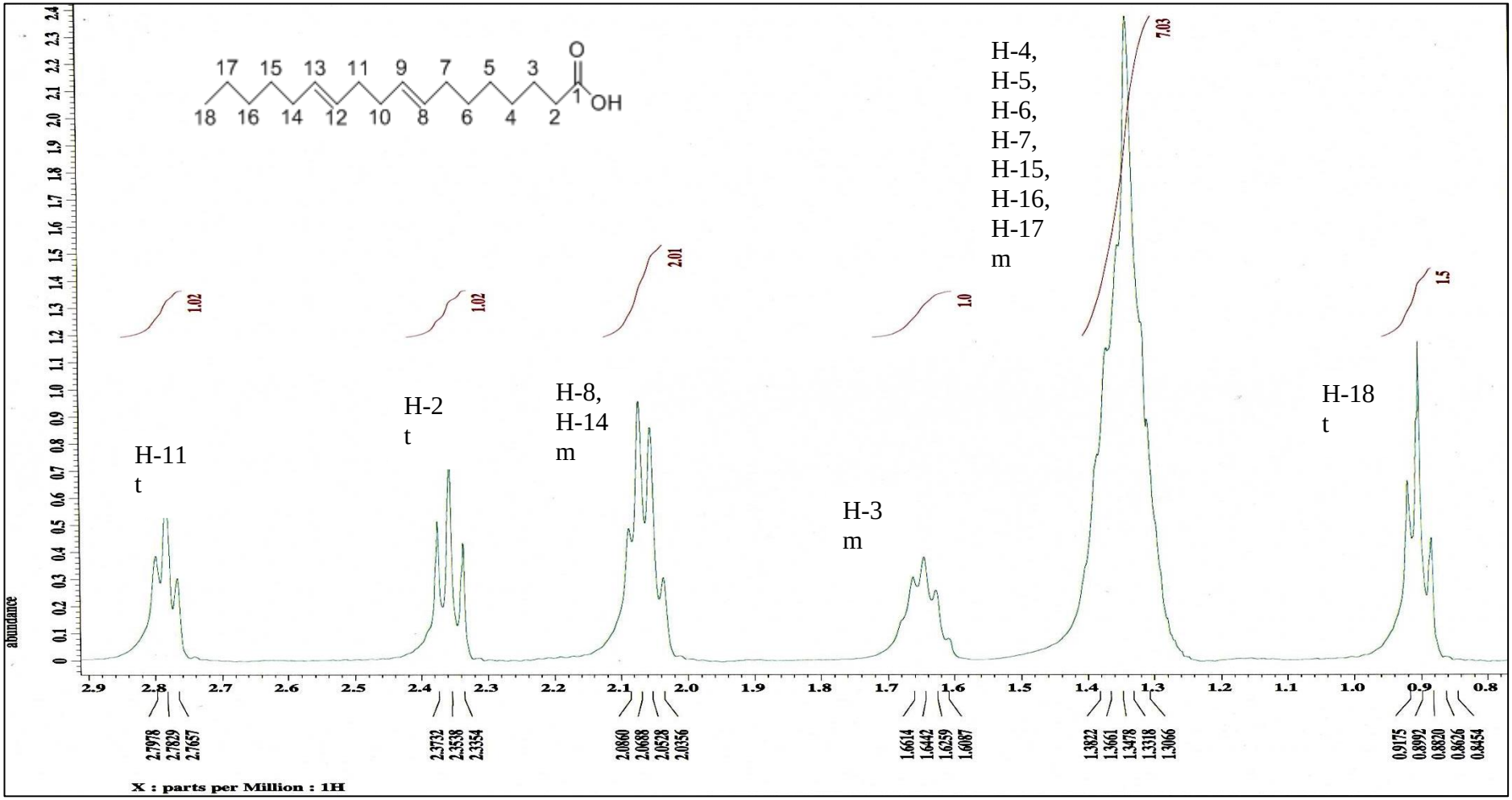


Figure 4.7a: The ^{1}H NMR 400 Hz of linoleic acid in $CDCl_3$ (ppm) (continued)

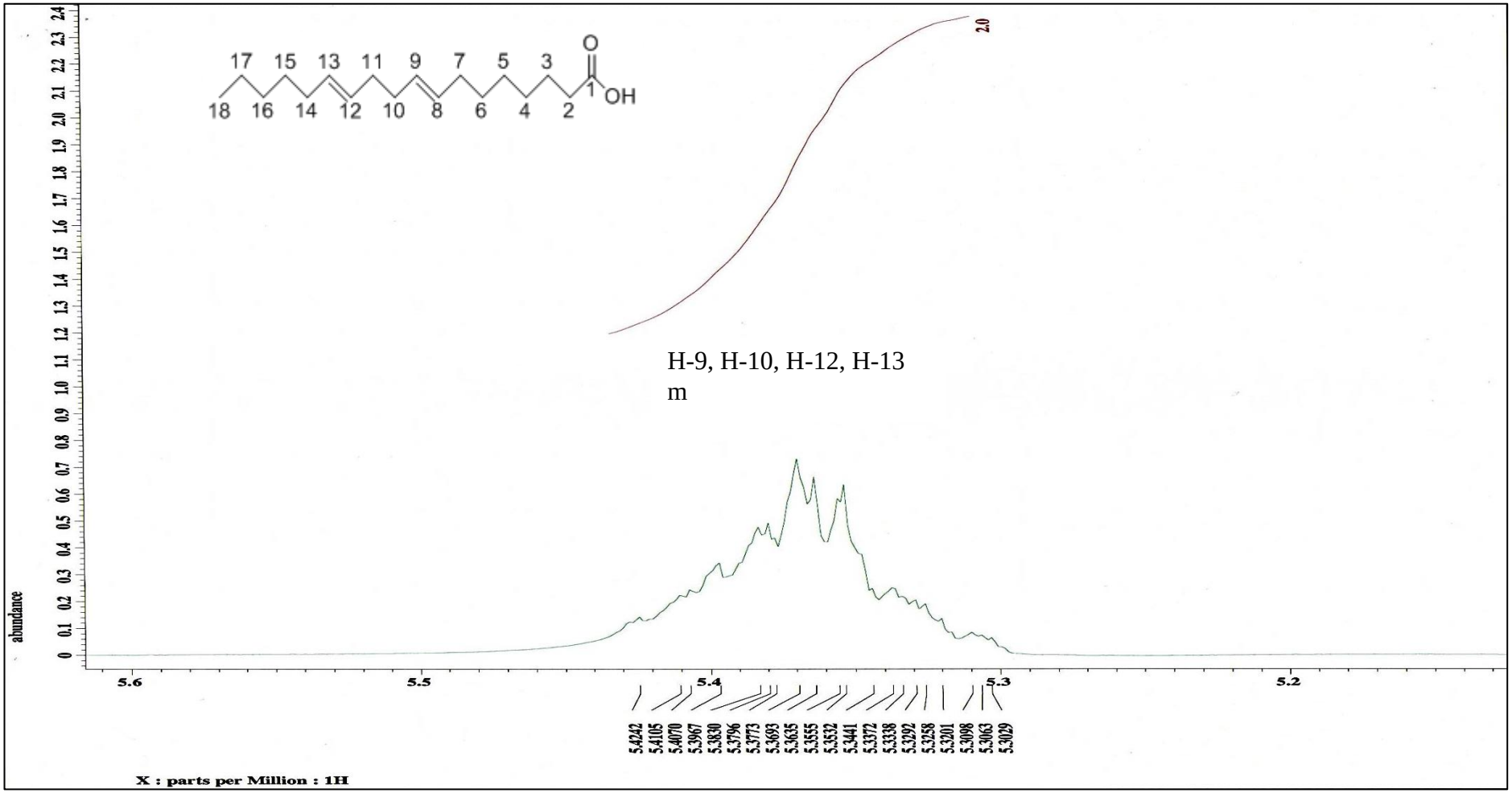


Figure 4.7a: The ^{1}H NMR 400 Hz of linoleic acid in $CDCl_3$ (ppm) (continued)

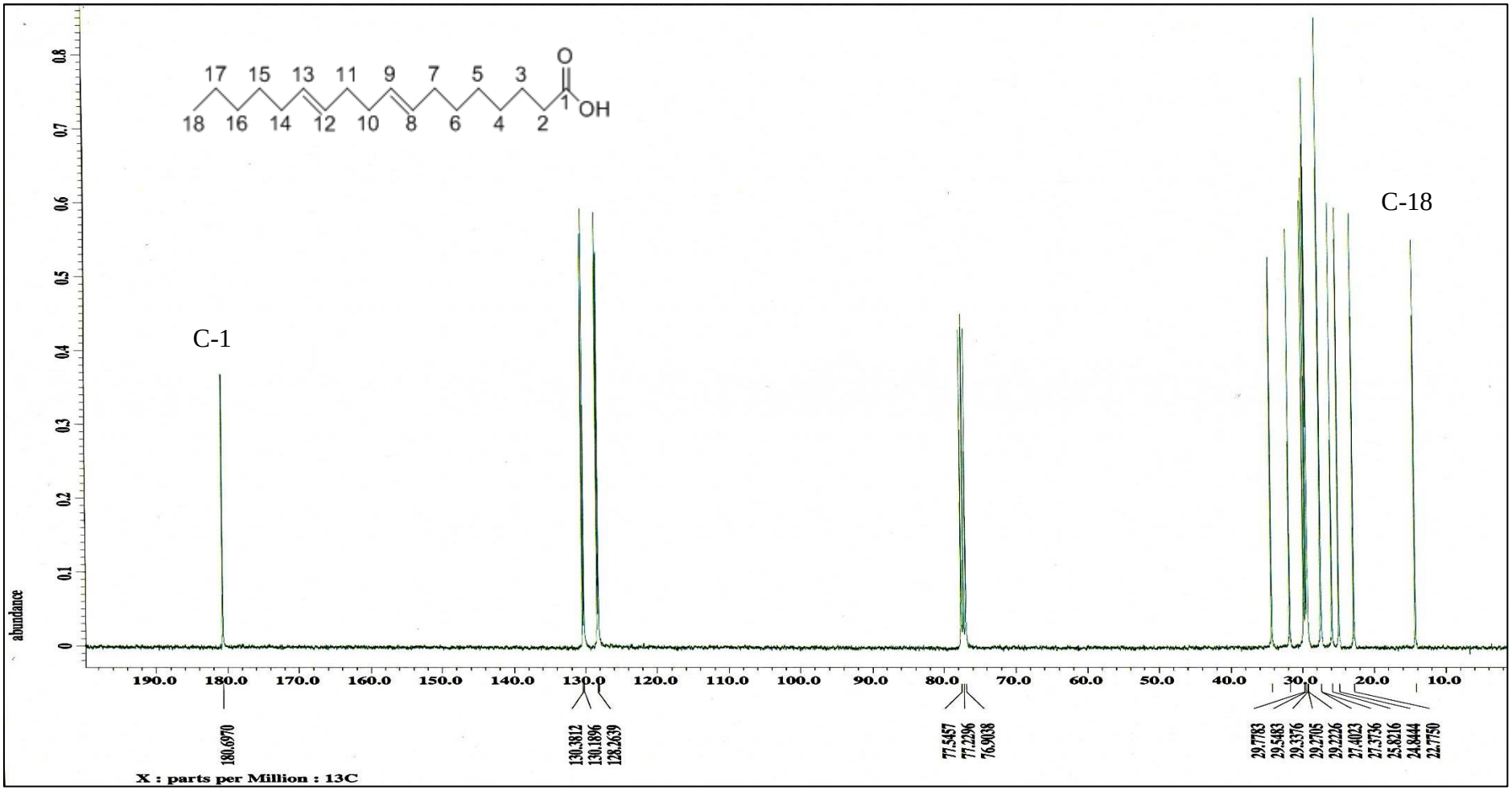


Figure 4.7b: The 13C NMR 400 Hz of linoleic acid in $CDCl_3$ (ppm)

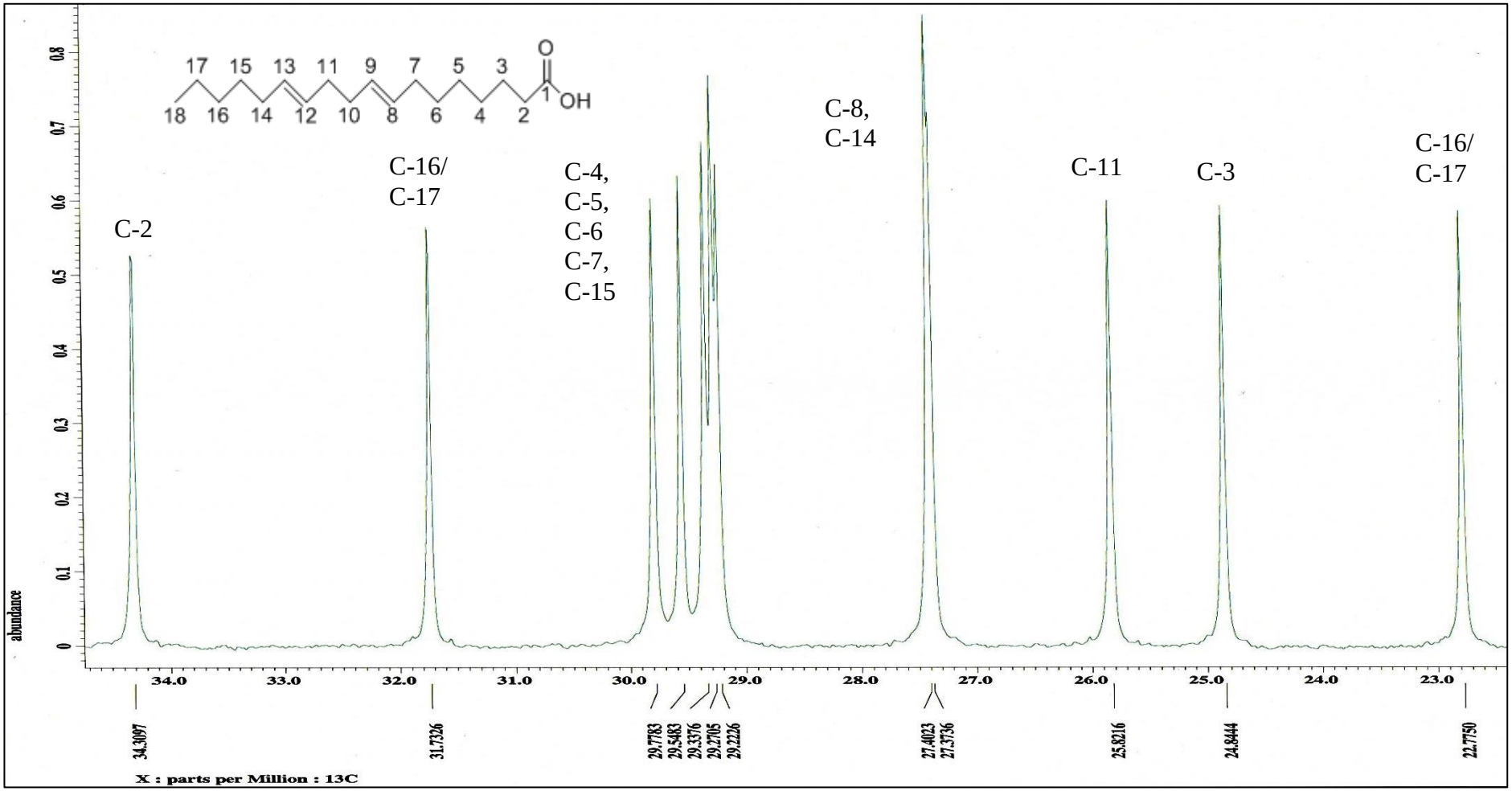


Figure 4.7b: The 13C NMR 400 Hz of linoleic acid in $CDCl_3$ (ppm) (continued)

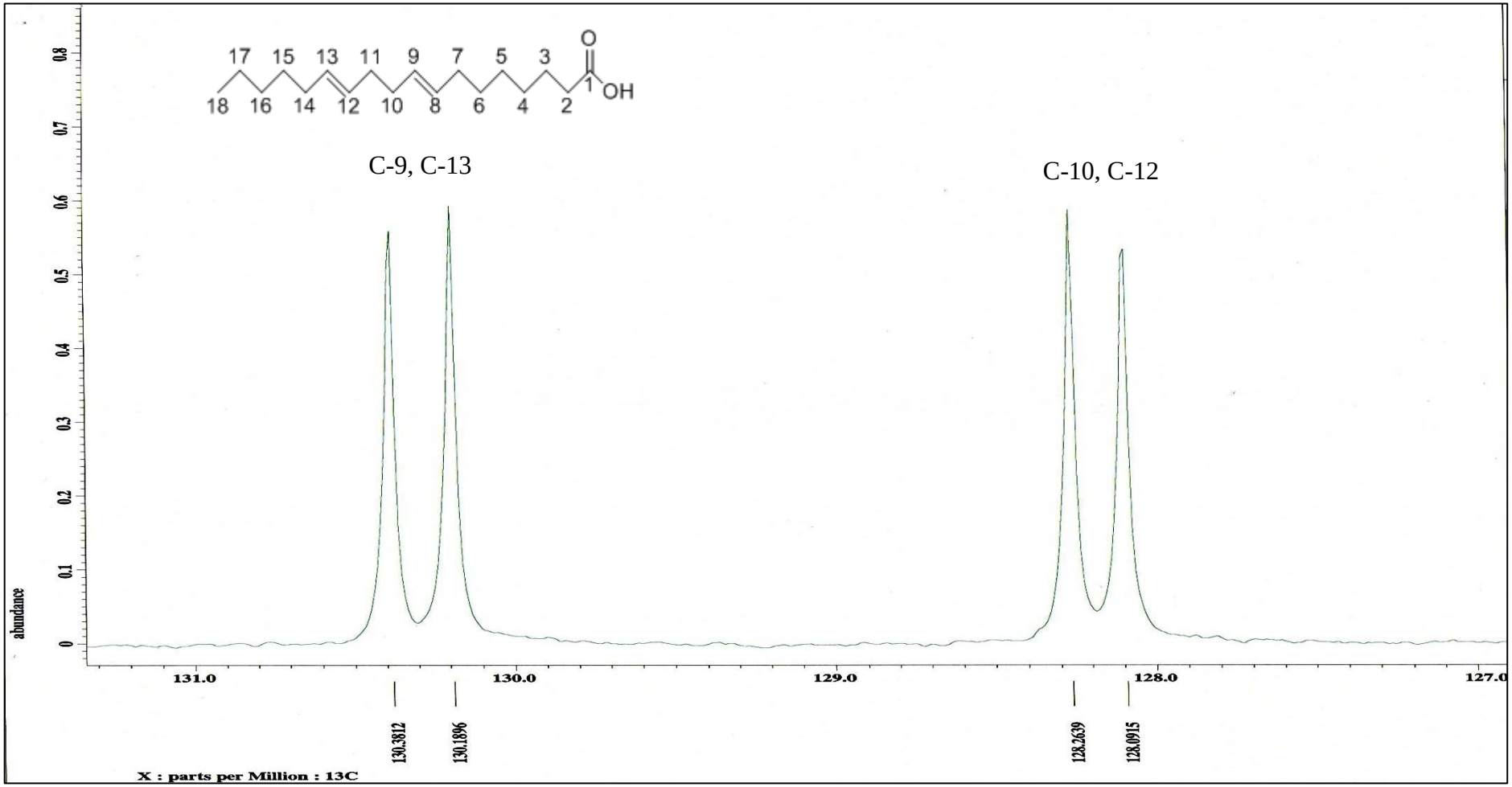


Figure 4.7b: The 13C NMR 400 Hz of linoleic acid in $CDCl_3$ (ppm) (continued)

Table 4.11: The ^{1}H and 13C NMR 400Hz data of palmitic acid

Position	δH[a]	δC
1		180.2
2	2.35 *t* (7.3)	22.9
3	1.64 *m*	32.2
4	1.26 *m*[b]	29.3[c]
5	1.26 *m*[b]	29.3[c]
6	1.26 *m*[b]	29.5[c]
7	1.26 *m*[b]	29.5[c]
8	1.26 *m*[b]	29.6[c]
9	1.26 *m*[b]	29.6[c]
10	1.26 *m*[b]	29.7[c]
11	1.26 *m*[b]	29.7[c]
12	1.26 *m*[b]	29.8[c]
13	1.26 *m*[b]	29.9[c]
14	1.26 *m*[b]	24.9[d]
15	1.26 *m*[b]	34.3[d]
16	0.89 *t* (6.4)	14.3

[a]Coupling constants (J) in Hz are indicated in parentheses
[b]Overlapping signals
[c,d]Chemical shifts are interchangeable

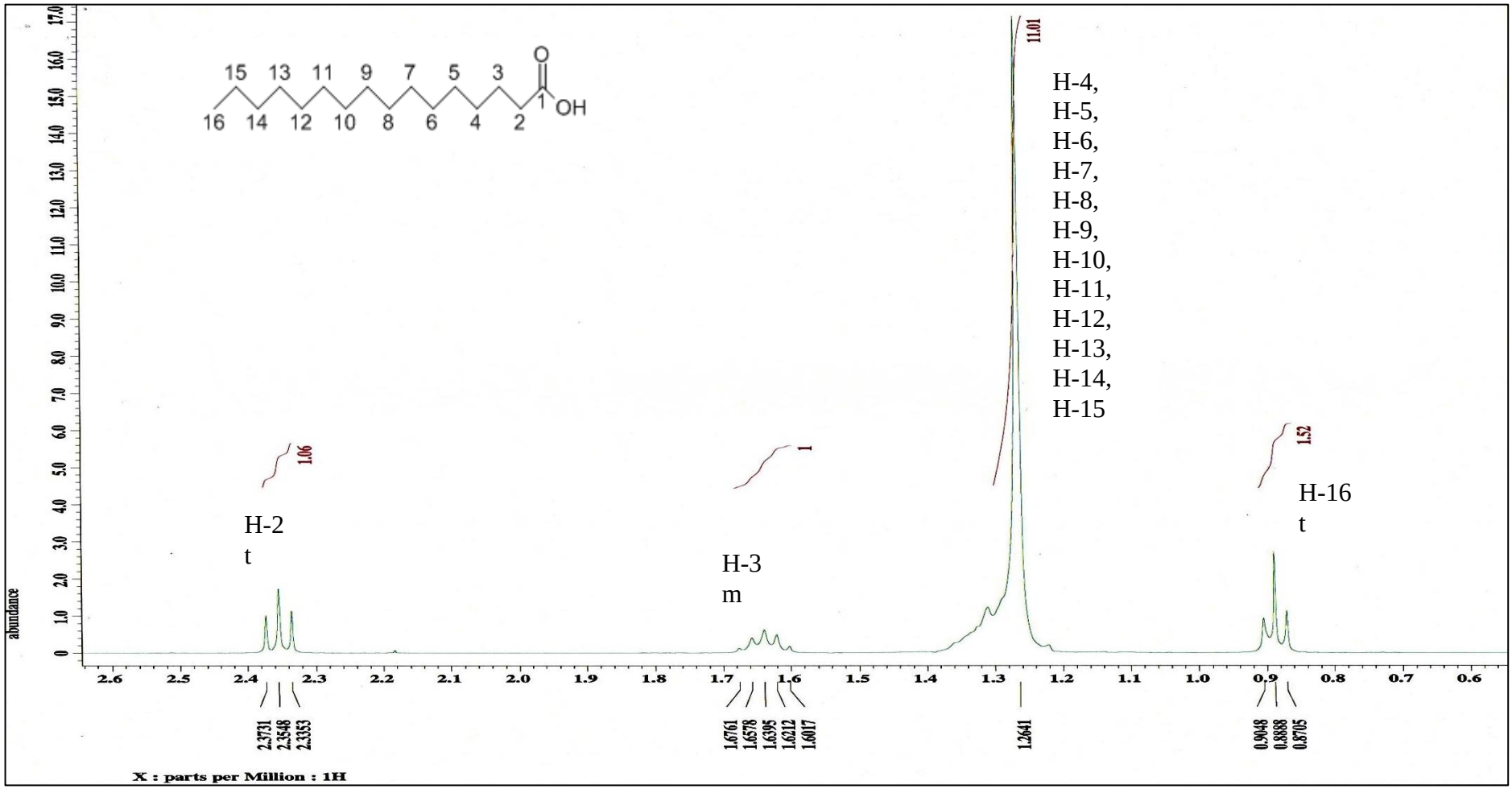


Figure 4.8a: The ^{1}H NMR 400 Hz of palmitic acid in $CDCl_3$ (ppm)

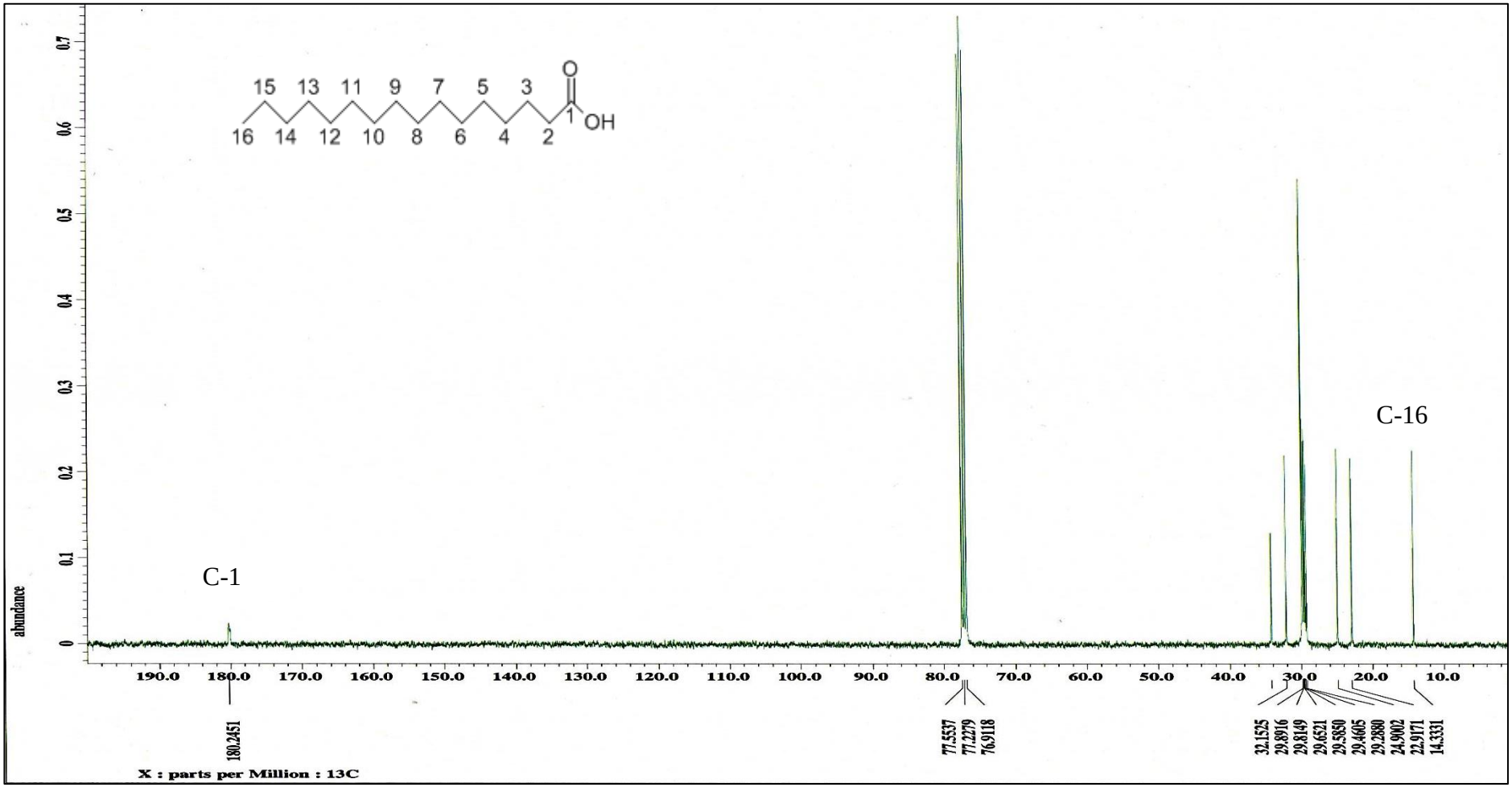


Figure 4.8b: The 13C NMR 400 Hz of palmitic acid in $CDCl_3$ (ppm)

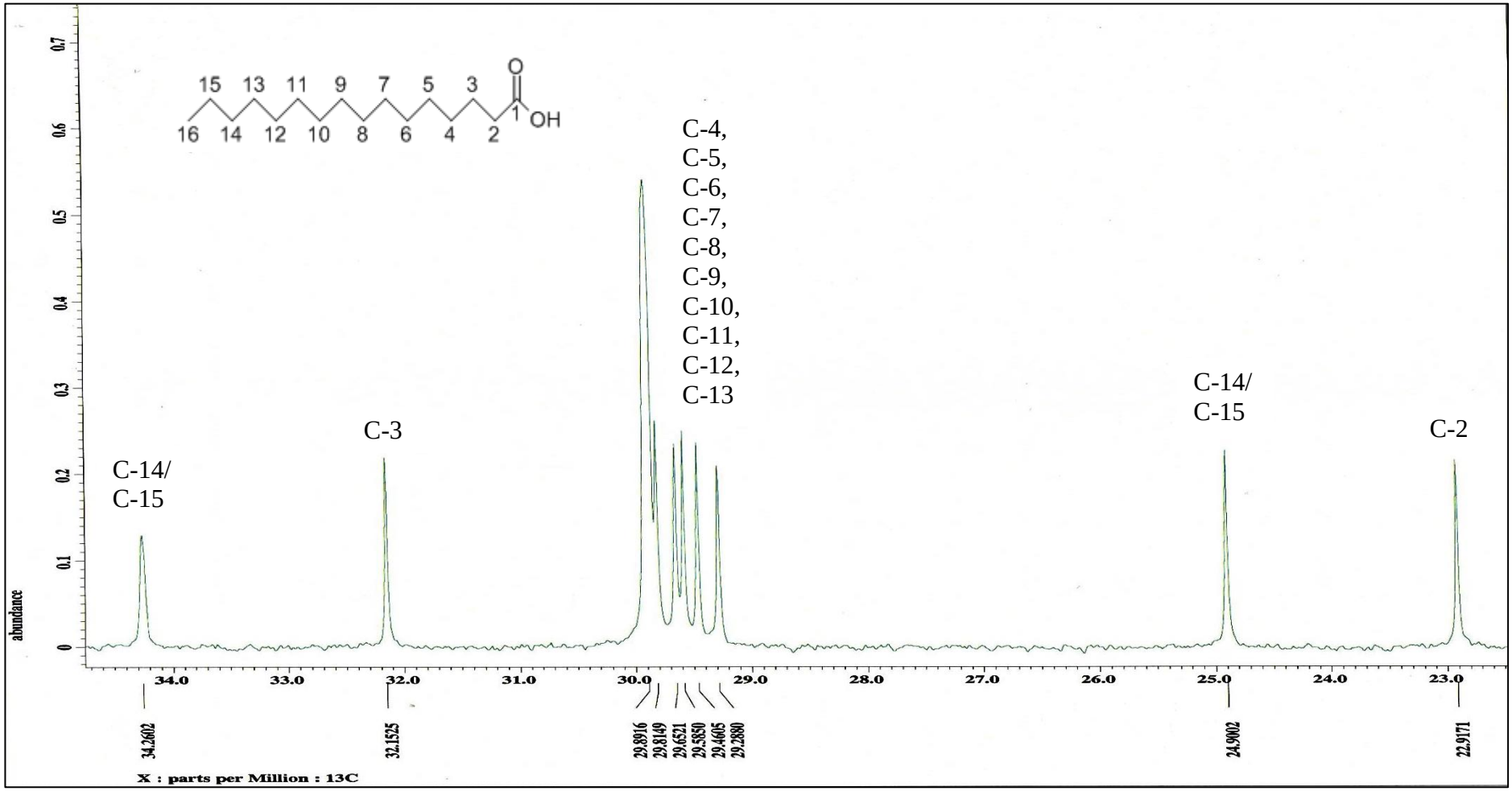


Figure 4.8b: The 13C NMR 400 Hz of palmitic acid in $CDCl_3$ (ppm) (continued)

4.7 Mass spectrometry data of metabolites from *Saccardoella rhizophorae*

The mass of linoleic acid was obtained as 280.2402 with mass of negative ions $(M-H)^-$ at 279.233 and mass of positive ions $(M+H)^+$ at 281.2475. The mass of palmitic acid was obtained as 256.2402 with mass of negative ions $(M-H)^-$ at 255.233 and mass of positive ions $(M+H)^+$ at 257.2475. The LCMS spectrums of linoleic acid and palmitic acid are shown is Figure 4.9a and 3.9b respectively.

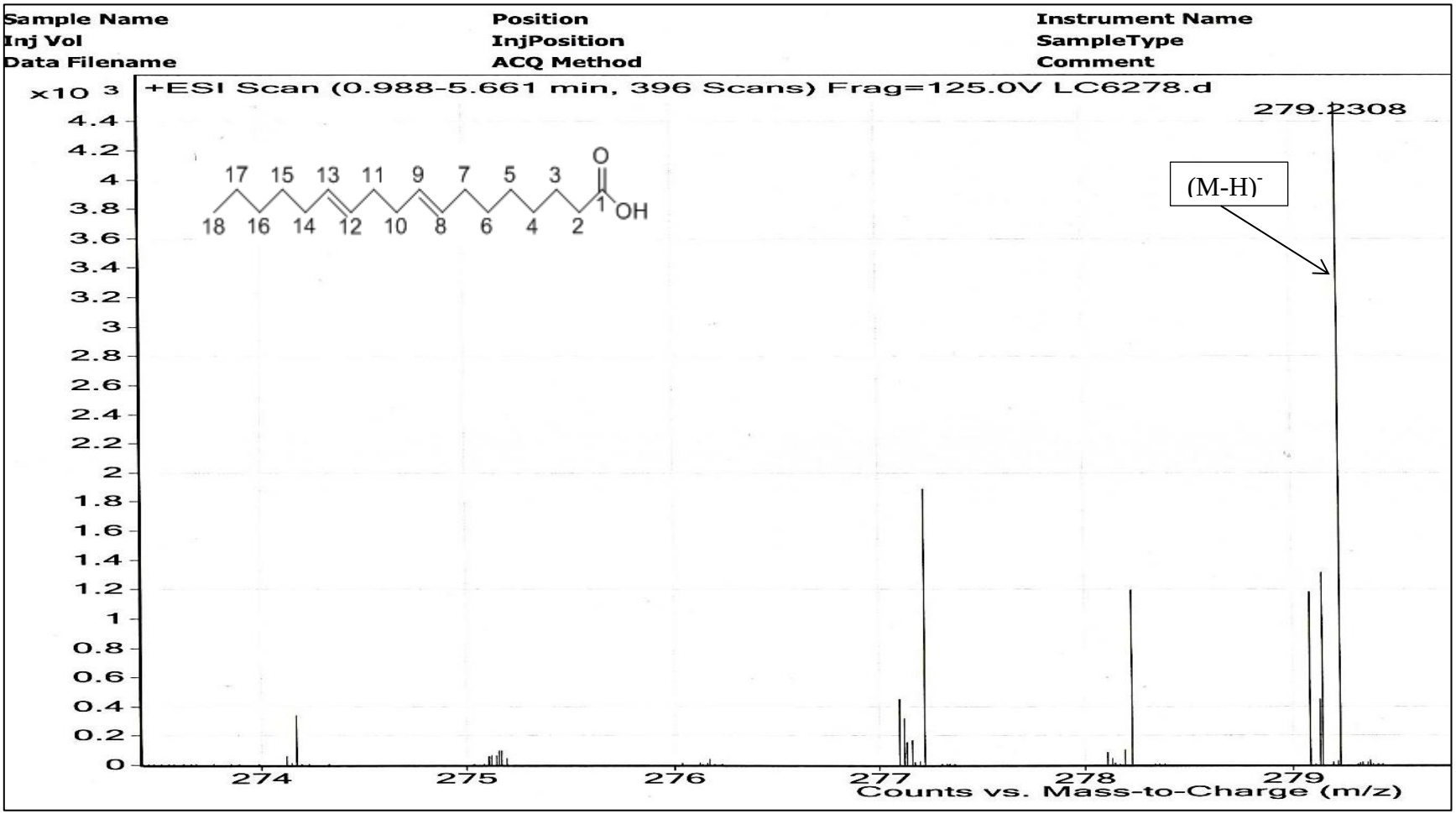


Figure 4.9a: LCMS spectrum of linoleic acid

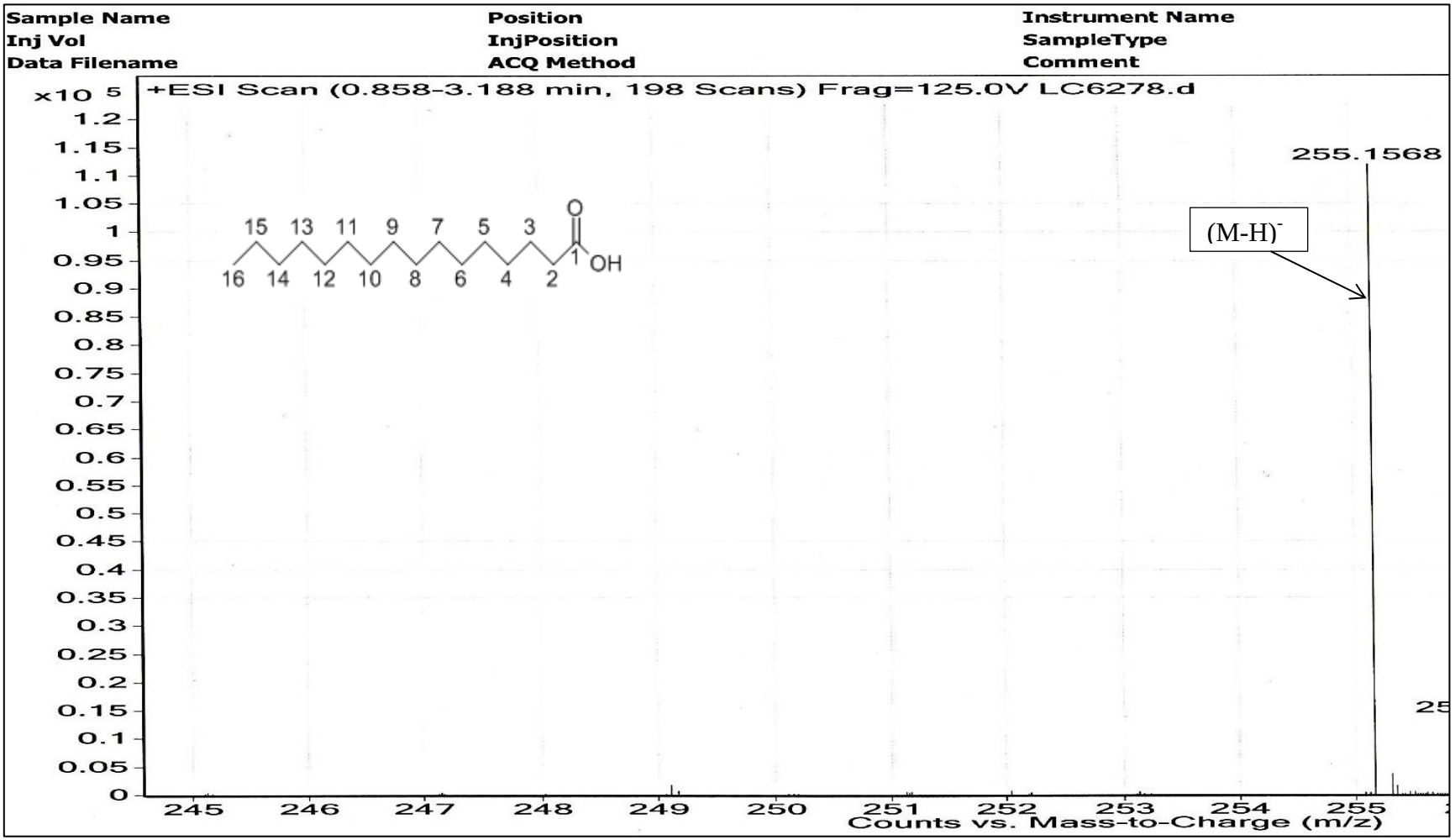


Figure 4.9b: LCMS spectrum of palmitic acid

4.8 Infrared (IR) spectroscopy data of metabolites from *Saccardoella rhizophorae*

Infrared (IR) spectrum of linoleic acid is shown in Figure 4.10a while the IR spectrum of palmitic acid is shown in Figure 4.10b. Both linoleic acid and palmitic acid are chemically classified as carboxylic acids which give them both similar stretch and bending patterns. Both IR spectrums have characteristic O-H stretch from 3300 to 2500 cm^{-1}. The normal ranges of stretches and bends for functional groups in carboxylic acids are; C=O stretch at 1760 to 1690 cm^{-1}, C-O stretch at 1320 to 1210 cm^{-1}, O-H bend at 1440 to 1395 cm^{-1} (Max *et al.*, 2004). Based on the IR spectrum of linoleic acid in Figure 4.10a, there is strong O-H stretch from 3300 to 2500 cm^{-1} with C=O stretch at 1747.32 cm^{-1}, O-H bend at 1325.25 cm^{-1}, and C-O stretch similarly at 1325.25 cm^{-1}. As for the IR spectrum of palmitic acid in Figure 4.10b, there is a strong O-H stretch from 3300 to 2500 cm^{-1} with C=O stretch at 1713 cm^{-1}, O-H bend at 1411.07 and 1378.28 cm^{-1} and C-O stretch at 1212.17, 1260.87 and 1276.75 cm^{-1}.

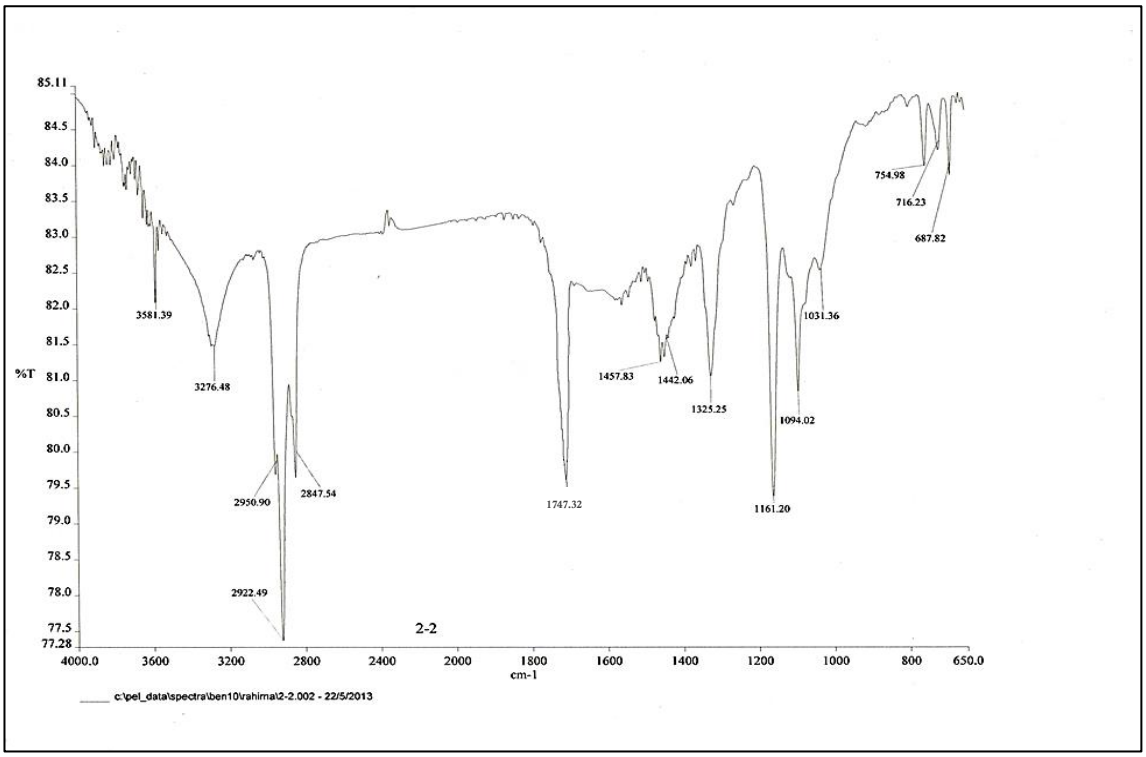


Figure 4.10a: IR spectroscopy data of linoleic acid

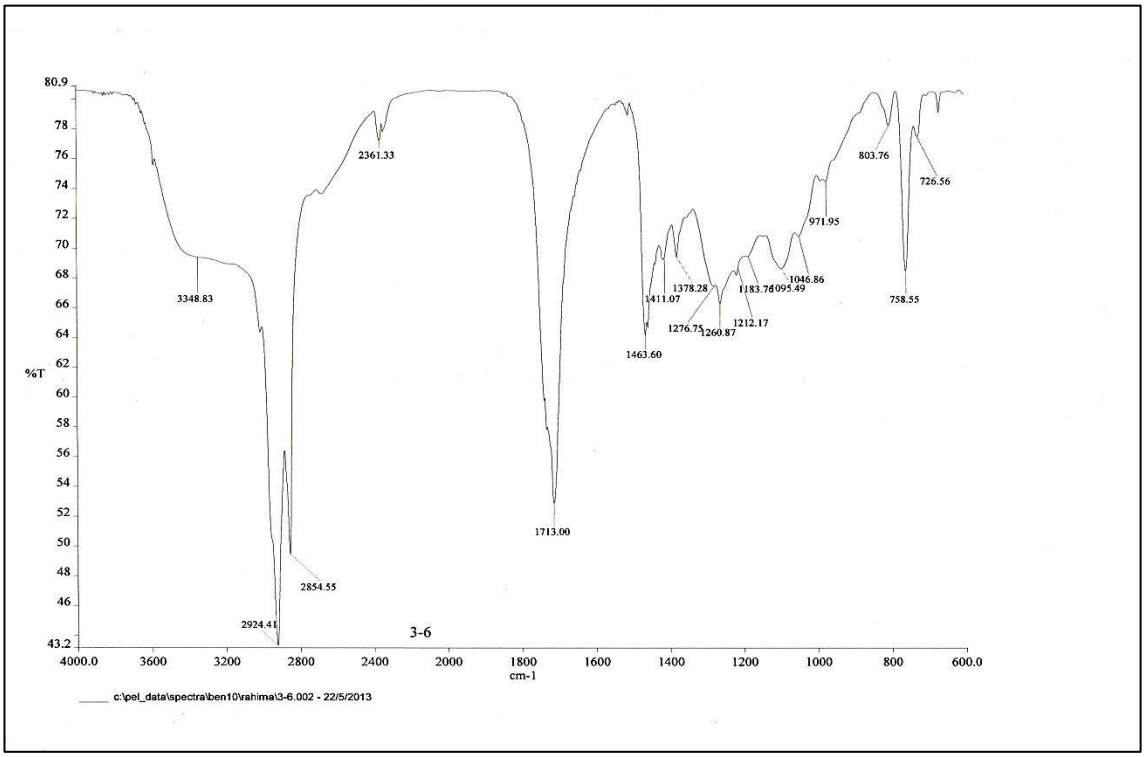
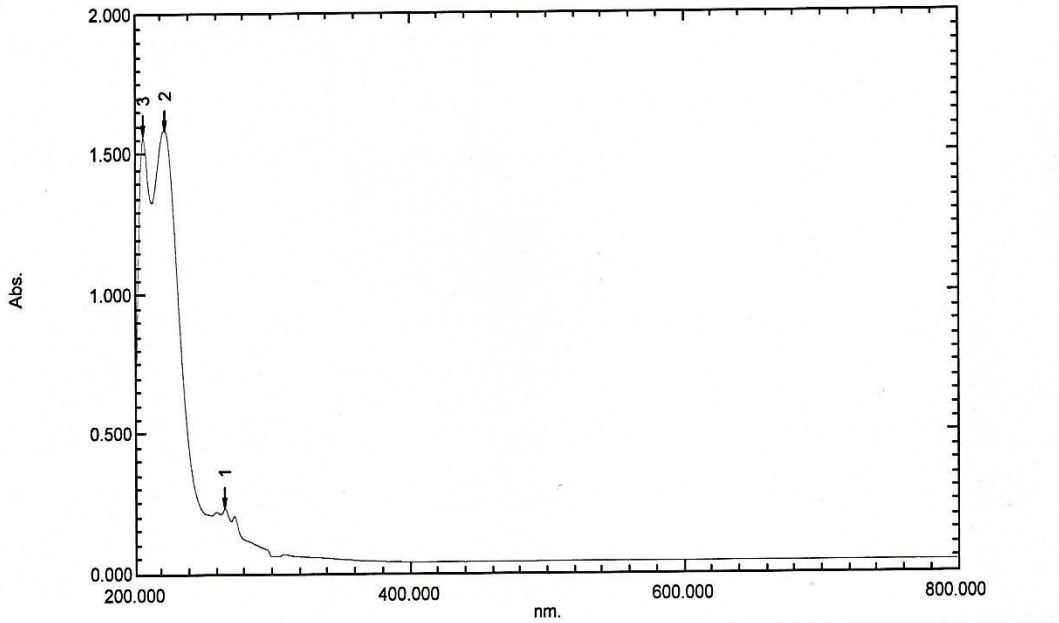


Figure 4.10b: IR spectroscopy data of palmitic acid

4.9 Ultraviolet (UV) spectroscopy data of metabolites from *Saccardoella rhizophorae*

The UV absorbances of bioactive compounds are shown in Figures 3.11a and 3.11b. The UV absorbance data of linoleic acid is displayed in Figure 4.11a while the data for palmitic acid is shown in Figure 4.11b. The standard absorbance range of absorbance for carboxylic acids is usually at 200 to 210 nm (Weishaar *et al.*, 2003). Both linoleic acid and palmitic acid exhibited maximum absorbance at 206 and 204 nm respectively, thus justifying that they are indeed carboxylic acids.

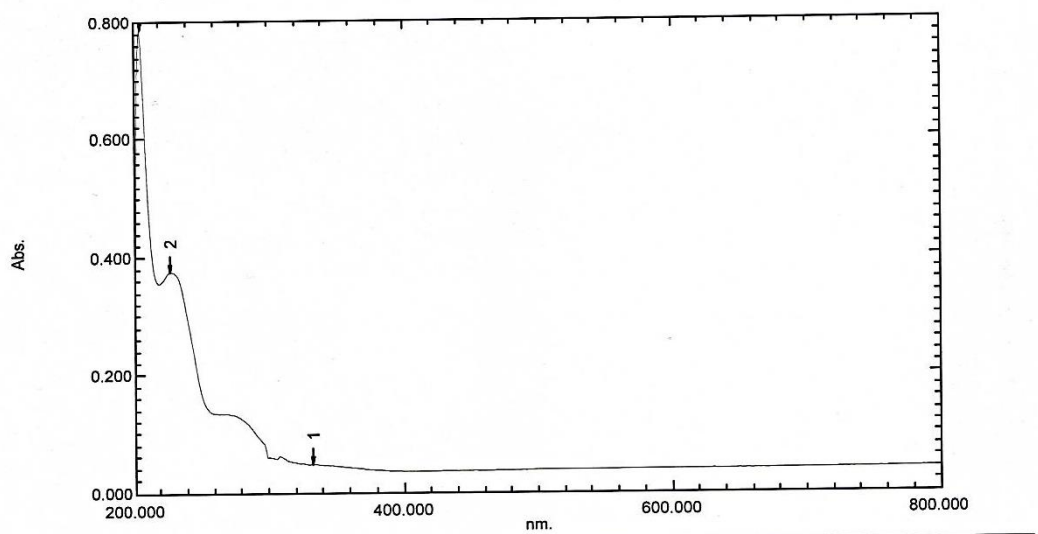


Measurement Properties

Wavelength Range (nm.):	200.000 to 800.000
Scan Speed:	Fast
Sampling Interval:	1.0
Auto Sampling Interval:	Disabled
Scan Mode:	Auto

No.	P/V	Wavelength	Abs.	Description
1	↑	265.000	0.234	
2	↑	221.000	1.586	
3	↑	206.000	1.570	

Figure 4.11a: UV spectroscopy data of linoleic acid



Measurement Properties

Wavelength Range (nm.):	200.000 to 800.000
Scan Speed:	Fast
Sampling Interval:	1.0
Auto Sampling Interval:	Disabled
Scan Mode:	Auto

No.	P/V	Wavelength	Abs.	Description
1	↑	332.000	0.048	
2	↑	226.000	0.375	
3	↑	204.000	0.786	

Figure 4.11b: UV spectroscopy data of palmitic acid

CHAPTER 5

DISCUSSION

5.1 Significance of molecular methods in the identification of endophytic fungi from *Ipomoea pes-caprae* and *Vitex rotundofolia*

Endophytic fungi are commonly described as fungi that completes at least a part of their life cycle inside plant tissues without causing any harm to the host plant (Linnakoski *et al.*, 2012). Some reports have also suggested the possibility of endophytic fungi having evolved from pathogens having latent phases within their life cycles thus justifying as to why these fungi do not cause harm to their host plants (Linnakoski *et al.*, 2012). The identification and diversity studies of fungi were traditionally carried out based on the morphological characteristics of the sexual and asexual fruiting bodies of the fungi. Pang *et al.* (2005) however debated that this mode of identification alone is insufficient due to reasons such as, inability of some fungi to grow in culture especially under laboratory conditions, different sporulation rates, multiple life cycle forms and also the difficulties in distinguishing species with similar morphologies. This is where molecular techniques came to play an essential role towards a more reliable method of fungal identification. Keeping the above statements in addition to the absence of fruiting bodies in consideration, the endophytic fungi isolated in the present study were identified using molecular techniques.

Prior to this study, there have been no reports of endophytic fungi isolated from the leaves of *V. rotundifolia*. Studies by Khan *et al.* (2012) have only reported plant growth promoting endophytic fungi from the roots of *V. rotundifolia*. As for *I. pes-caprae*, there have been reports such as the one from Beena *et al.* (2000) in which the endophytic fungi from the roots of *I. pes-caprae* were investigated. The only endophytic

fungus reported from the leaves of *I. pes-caprae* is *Fusarium oxysporum* (Guo *et al.*, 2013). Thus overall, there has been minimal work done to investigate the diversity of endophytic fungi from the leaves of *V. rotundifolia* and *I. pes-caprae*.

The 1940's marked the start for the study of fungal genetics due to the nature of fungal genes that made them ideal models (Hooley *et al.*, 2006). According to Hooley *et al.* (2006), fungal diversity in marine habitats can be assessed by performing PCR of repetitive DNA sequences, in particular sequences that encodes small or large ribosomal subunits and their associate ITS regions. Anderson *et al.* (2007) reported that the last decade has seen a marked progress in fungal ecology due to the use of molecular methods to analyse the variation of sequences within rRNA genes and their associated spacer regions. Fungal diversity in natural environments can be assessed using certain molecular techniques namely; denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphism (SSCP), terminal restriction fragment length polymorphism (T-RFLP), amplified rDNA restriction analysis (ARDRA), amplified ribosomal intergenic spacer analysis (ARISA) and cloning (Pang *et al.*, 2005). However most of the techniques mentioned above are more suitable when the sample in concern is a mixture of fungi strains usually involving direct environmental samples.

The objective of the present study was to identify the endophytic fungi strains to the level of its genus based on the database, therefore only the ITS region was analysed. Polymerase chain reaction (PCR) was employed to amplify and further compare the sequences of the ITS region incorporating the 5.8S rRNA fungal gene to identify the endophytic fungal isolates. The ITS primers work well on DNA from individual organisms thus making this technique possible as all the fungal strains in the present study were pure cultures (Prewitt *et al.*, 2008). The rRNA genes are a multi-copy gene family that are arranged in tandem repeats where each major repeat contains coding

regions of the primary rRNAs and non-coding spacer regions. In ecological studies of fungi, the genes encoding 18S rRNA and 25/28S rRNA, and the ITS regions incorporating the 5.8S rRNA gene are the target regions within the rRNA gene cluster normally studied upon (Anderson *et al.*, 2007). In comparison to other rRNA gene clusters, the ITS region is extremely variable and have evolved at a slow pace thus allowing the detection of a higher number of phylotypes (Prewitt *et al.*, 2008). Although highly conserved within most species but the ITS region is variable between species thus making it suitable in taxonomy studies (Prewitt *et al.*, 2008). The forward ITS 5 and reverse ITS 4 primers in the present study were to target the ITS regions flanking the 5.8S rRNA, similar to studies by Guo *et al.* (2003). According to Toju *et al.* (2012), primers designed for ITS analysis should match the sequences of 99% of fungal lowest taxonomic units (LTUs) to enable a thorough and unbiased DNA barcoding. Both primers ITS 5 and ITS 4 exhibits broad coverage of fungal lowest taxonomic units (LTUs) with the coverage of ITS 4 as broad as that of any other primer including new and improvised primers (Nikolcheva *et al.*, 2004). Nikolcheva *et al.* (2004) reported that the forward primer ITS 5 is capable of amplifying all Fungi thereby justifying the choice of this primer in the present study.

When compared to other primers, the ITS primers provided higher hit rates for fungal sequences in BLAST searches due to the abundance of ITS data in the GenBank (Pang *et al.*, 2005; Arnold, 2007). However the limitations with using the ITS data as a tool for identification is that its accuracy is limited to comparing distantly related organisms (Lamothe *et al.*, 2002). Therefore in phylogenetic studies of fungi, generally more than one gene is studies upon in determining the identity and relatedness of fungi strains. There have also been studies that indicate the ITS data alone is insufficient for identifications due to the fact that some fungi are not represented in the GenBank and therefore fungi strains may be misidentified due to the lack this lack of taxonomic

information (Arnold, 2007). A study by Prewitt *et al.* (2008) on the phylogenetic of wood decay fungi reported that the ITS sequence alone could not resolve the differentiation between certain species of basidiomycetes. Studies such as those by Ho *et al.* (2012) employed the analysis of sequences from two different genes to obtain a more accurate identification of endophytic fungi strains. However in this study we merely focused on basic identification as the objective of this study was more towards investigation of the bioactivity of the fungi thereby justifying the use of only one gene in the identification of the fungi.

5.2 Antimicrobial susceptibility testing of marine derived fungi extracts

The disk diffusion test is usually the gold standard by the Clinical and Laboratory Standards Institute (CLSI) in qualitative antimicrobial susceptibility testing. This technique relates the diameter of zone of growth inhibition around antibiotic disks to the susceptibility of the isolates tested. The plug assay technique by Hoskisson *et al.* (2001) and Ezra *et al.* (2004) was chosen due to its inexpensive and fast approach to detect the antibacterial bioactivity of both marine derived manglicolous and endophytic fungi. In the present study, the plug assay technique served as a primary method to downstream the large amount of fungi isolates to achieve a systematic way of identifying the fungi isolates with promising antibacterial activity for further analysis. This assay requires no prior extraction of fungal secondary metabolites which eliminated the time and consumables consumed in doing so. However, this assay has its own share of disadvantages such as; the antibacterial activity results obtained are only qualitative and not quantitative measurements (Zainuddin *et al.*, 2008). This is because the plug assay only assesses the mere exhibition of antibacterial activity without taking into account the concentration of the fungi extracts as a parameter in the display of antibacterial activity.

The plug assay does not employ the need for prior extraction of fungal secondary metabolites thus there is no way of ensuring the presence and concentration of secondary metabolites secreted on the medium. There may be a possibility that the fungi that exhibited no antibacterial activity in the plug assay, may exhibit antibacterial activity when cultured in broth under specific conditions and their secondary metabolites extracted with appropriate solvent systems and then further tested, similar to studies by Zainuddin *et al.* (2008). To ensure that the fungal culture media did not influence the antibacterial activity displayed by the fungi, the exchange of a plug of sterile PDA instead of fungal culture was used as the negative control in the plug assay.

The seven test bacteria used in this study comprised of five Gram positive and two Gram negative bacteria. All the bacterial strains chosen were known causes of nosocomial infections affecting the medical world. Test bacteria were also chosen based on four criterias' such as Gram negative or positive, morphology, endospore and non-endospore forming bacteria, enterobacteriaceae and non-enterobacteriaceae. These criteria served as a tool towards understanding if the antibacterial activity displayed by the fungi were selective towards or against a given morphological or physiological trait of the test bacteria. Focusing on the first criteria, the Gram positive bacterial strains consisted of *Staphylococcus aureus*, *Bacillus cereus*, *Bacillus subtilis*, *Micrococcus luteus* and *Enterococcus faecalis* while the Gram negative bacterial strains consisted of *Escherichia coli* and *Pseudomonas aeruginosa*. Taking into account of the second criteria which is morphology, the test bacteria were selected based on their shapes. *S. aureus*, *M. lutues*, *E. faecalis* and *E. coli* are all spherical/cocci shaped while *B. cereus*, *B. subtilis*, *P. aeruginosa* are rod shaped bacteria. Thirdly in terms of endospore formation, only *B. cereus* and *B. subtilis* are endospore forming bacteria while the other five are non-endospore forming bacteria. Lastly, as for bacteria from the enterobacteriaceae family, the bacterium *E. coli* belongs to this family.

One of the earliest techniques of antimicrobial susceptibility testing was the macrobroth method. This technique provides quantitative results but one of the principal disadvantages of this method is that it is rather tedious and requires the use of large amounts of reagents and space. The miniature version of the macrobroth method is the broth microdilution technique by Eloff, (1998) which is carried out in 96 well plates and requires relatively smaller amounts of reagents and space in comparison. Another advantage of the broth microdilution method is that this technique can also be performed using both manual and automated systems whereby hundreds of 96 well trays can be prepared from a single master set of dilutions in a short period of time

(Jorgensen *et al.*, 2009). The broth microdilution assay was used to screen the antibacterial activity of EA crude extracts of selected marine derived fungi in the present study. This assay required prior extraction of the secondary metabolite crude from the fungi using EA. Ethyl acetate was chosen as the solvent of extraction mainly due to its high polarity index of 3.9 but yet with a solubility of 8.7% in water. Due to fact that the sample to be extracted was fermentation broth which is aqueous, the solvent of choice needed to be able to extract both polar and non-polar chemical compounds while at the same time be non-soluble in water to achieve clear separation and partition. The solvent EA has been a favoured solvent in the extraction of fungal secondary metabolites as reported in similar studies by Han *et al.* (2009) and Kjer *et al.* (2010).

In the broth microdilution assay, the extraction of sterile PDB broth with EA served as a negative control to prove that the bioactivity displayed was only with respect to the fungal secondary metabolites and not the constituents of the broth. From the results obtained, the sterile broth extract showed no antibacterial activity against all test bacteria. The antibiotics chloramphenicol and penicillin G served as positive controls in this bioassay. The antibiotics in this study possessed two varying antibacterial mechanisms. Penicillin G is a β-lactam antibiotic that inhibits the formation of peptidoglycan cross-links in the bacterial cell wall while chloramphenicol acts as a protein synthesis inhibitor. This study employed two different antibiotics as controls as not all bacterial strains are susceptible to one class of antibiotic and thereby the use of only one antibiotic may provide a false assumption that the bacterial strain is antibiotic resistant when in actual fact, the bacterial cell does not provide suitable target for the action of the antibiotic used.

The broth microdilution technique was employed to obtain a more quantitative data of the antibacterial activities of marine derived fungi based on preliminary analysis

results through the plug assay. Although the disc diffusion assay has always been a favoured choice in antimicrobial susceptibility testing, this assay is only a semi-quantitative assay in comparison to the broth microdilution assay which is more quantitative (Domig *et al.*, 2007).

5.3 Endophytic fungi as producers of bioactive metabolites

The two host plants in the present study have not been fully exploited in terms of their endophytic fungi. There have been no studies on the bioactivities of endophytic fungi from the plant *V. rotundifolia* while there was only one study pertaining to the bioactivity of endophytic fungi from *I. pes-caprae*. A study by Guo *et al.* (2012) reported the isolation of exopolysaccharide from the endophytic fungus *F. oxysporum* isolated from the plant *I. pes-caprae*. However the study mentioned above was not related to antibacterial bioactivity from the endophytic fungi. Other than that, no other reports have been published from studies on the bioactivity of endophytic fungi from these 2 plants.

Strobel *et al.* (2003) stated that the rationale behind endophytic fungi host selection for bioactivity studies should be based on, firstly the uniqueness of the host's environment such as harsh environments that require specific survival strategies and secondly, plants that possess an ethno botanical history. Endophytic fungi isolated from plants exploited for their medicinal values have been known to produce interesting compounds with profound activities (Suryanarayan *et al.*, 2009). Both the marine associated plants chosen in this study namely; *V. rotundifolia* and *I. pes-caprae*, have been reported to be exploited as herbal remedies in traditional medicine which could have played an important role in the display of their antibacterial activities.

The seed of *V. rotundifolia* has been reported to be used for cold remedies and headaches by the Japanese and Chinese; it is used as a raw material for Chinese traditional medicine. Some diterpenoids and sesquiterpenoids have been obtained from this plant as well as some insecticidal substances (Kawazoe *et al.*, 1999). A study by Ono *et al.* (1998), reported the discovery of 7 phenolic compounds with strong antioxidant activities as well as the isolation of a new labdane-type diterpene glycoside

from *V. rotundifolia*. In Korea, this plant is well known as “Man Hyung Ja” and is used by natives against headaches related to upper respiratory infection as well as various allergic diseases (Shin *et al.*, 2000). The analgesic properties of *V. rotundifolia* have also been reported and scientists have isolated polymethoxyflavonoid, a compound with the ability to inhibit proliferation of lymphocyte and the growth of some cancer cell lines (Ko *et al*., 2000).

The plant *I. pes-caprae* in the present study also has well known ethnobotanical history where the genus *Ipomoea* from the Convolvulaceae family is a known source of complex resin glycosides with interesting bioactivities. *I. pes-caprae* is used as an herbal drug in Mexico mainly in the treatment of kidney complaints, digestive disorders, hypertension, arthritis, rheumatism, skin infections and other inflammatory conditions. Furthermore, nine compounds have been isolated from *I. pes-caprae* with antibacterial activity (Martinez *et al.*, 2010). Umamaheshwari *et al.* (2012) reported that this plant has also been widely explored for its antioxidant, analgesic, anti-inflammatory and good anti-nociceptive properties.

The endophytic fungi from *V. rotundifolia*; *C. eragrostidis* (JN943448.1), *Curvularia* sp. (GQ1874733.1), *G. mangiferae* (KC686598.1), *Paecilomyces* sp. and *Phoma* sp. (JN207285,1) only displayed antibacterial activity against three of the seven test bacteria namely; *S. aureus*, *B. cereus* and *B. subtilis*. None of the endophytic fungi from *V. rotundifolia* exhibited antibacterial activity against the Gram negative bacteria in this study. This concludes a rather narrow range of antibacterial activity by the endophytic fungi from *V. rotundifolia* as the activity recorded covered only Gram positive bacteria. Results showed that the endophytic fungi isolated from the plant *I. pes-caprae* possessed prominent activity with broad spectrum against both Gram positive and negative bacteria compared to the endophytic fungi strains from *V. rotundifolia*.

This study reported that 20% and 3.33% of endophytic fungi from *I. pes-caprae* exhibited antibacterial activity against the Gram negative bacteria *E. coli* and *P. aeruginosa* respectively. Based on reports published, *I. pes-caprae* has been reported to possess antibacterial activity while no reports of antibacterial activity has been studied or reported from *V. rotundifolia*. Therefore the broad spectrum of antibacterial trait displayed by the plant *I. pes-caprae* could have been trait inherited by the endophytic fungi isolated from the plant. In a more recent report, Radic *et al.* (2012) and Kusari *et al.* (2012) characterized endophytic fungi as a "treasure chest of antibacterial substances"; further stressing that they possess the ability to produce same or similar bioactive compounds as the fungi's host plant.

The hypothesis of endophytic fungi being able to synthesize similar compounds as their host plants was initiated by the discovery of the revolutionary anticancer drug paclitaxel (taxol), from the endophytic fungi *Taxomyces andreanae* in 1993 (Stierle *et al.*, 1993). Taxol was previously isolated from the Pacific yew tree. Although the report by Stierle *et al.* (1993) is still regarded baseless by some scientists, there have been other reports where the active compound previously known to originate from a particular plant, is found to be also produced by the endophytic fungi of the same plant. For instance, the cerebral stimulant and vasodilator vincamine which was isolated from the plant *Vinca minor* was also isolated from the endophytic fungi of *V.minor* (Yin *et al.*, 2011). Another such report was the isolation of rhein, an antimicrobial and antitumor compound known to be from the Chinese medicinal plant *Rheum palmatum* L. from the endophytic fungi of the same plant (You *et al.*, 2013). Thus these findings further support the hypothesis that the activities demonstrated by the endophytic fungi from *I.pes-caprae* may be influenced by the antibacterial activities that this plant is known to possess.

Over the years, the studies on fungal secondary metabolites were based mainly on random activity screening. Recently, scientists have adopted a systematic approach in the discovery of metabolites of fungal origin with proven benefits to human life. According to Schulz *et al.* (2002) fungi that occupy certain ecological niche and possess characteristic metabolic interactions with their surrounding environment, may possess a greater potential for the synthesis of novel compounds. In line with the statement by Schulz *et al.* (2002), the collection site of *I. pes-caprae*, which is the west coast, Peninsular Malaysia, could have also played a role to influence the antibacterial activity exhibited by the endophytic fungi isolated from the plant; as justified by Agusa *et al.* (2005) stating that the Straits of Malacca (west coast, Peninsular Malaysia) is subjected to higher pollution rates due to industrialization and urbanization besides being the major shipping hub of the country. Studies by Yap *et al.* (2002) stated that the sediment samples analyzed from the west coast of Peninsular Malaysia reported high levels of heavy metal contamination; further justifying the high pollution rates in the stated area.

Overall, the antibacterial activity displayed by the endophytic fungi was found to be more pronounced against Gram positive in comparison to Gram negative bacteria. This finding is in line with other studies by Tong *et al.* (2011) and Buatong *et al.* (2011). Studies have shown that Gram negative bacteria are more resistant to antimicrobials due to the presence of the outer membrane permeability barrier which acts as a barrier to limit the influx of antimicrobials to their targets in the bacterial cell (Poole, 2001). Furthermore, Gram negative bacteria belonging to the Enterobacteriaceae family (eg: *E. coli*) have been proven as serious threats in nosocomial infections due to their spread of resistance (Paterson, 2006). Bacteria from the Enterobacteriaceae family have been reported to produce enzymes such as Extended Spectrum Beta Lactamase (ESBL) which are able to break down many commonly used antibiotics; which justifies

the resistance displayed by *E. coli* in this study with none of the only 20% of the endophytes from *I. pes-caprae* were active against this bacterium.

The ability of endophytic fungi to produce the same types of compounds as its host plant may provide an alternative strategy towards the sustainable production of certain pharmacologically important substances without exploiting or affecting the biodiversity of plants and in the same time reducing the market cost of the product. Besides this, fungi can be commercially cultivated under optimized fermentation conditions thereby leading to continuous and reproducible compounds. This is more effective than harvesting the same compounds from its host plant as the production of such compounds is dependent on the host's environment and as such the quantities and quality of compounds produced by the plant may differ according to different environments (Kusari *et al.*, 2012).

Certain endophytic fungi isolates in the present study exhibited promising antibacterial potential and may therefore be potential candidates for the discovery of new and novel antibacterial compounds. Endophytic fungi especially the ones isolated from the plant *I. pes-caprae* namely; *Minimidochium* sp. and *Bipolaris* sp. should be further analysed to elucidate their bioactive compounds through bioassay guided fractionation coupled with NMR studies.

5.3.1 Endophytic fungi and their antimicrobial activities

A number of endophytic fungi strains isolated in this study have also been reported from previous studies. The antimicrobial activities of these fungi have also been stated in several studies which may further justify the activities similar isolates exhibited in this study. From the 34 endophytic fungi isolated from both plants in this study, the fungi strain most commonly found was *Phoma* sp. consisting of almost 14.7% of the

endophytic fungi. Fungi from the genus *Phoma* are usually characterized as phytopathogens with the ability to cause losses in commercial crops (Liu *et al.*, 2003). Although usually isolated from the terrestrial habitats, *Phoma* sp. has also been isolated from the marine environment such as from marine microbial mats in the Bahamas (Liu *et al.*, 2003). Goldring *et al.* (2012) reported the isolation of *Phoma* sp. from the shell of a crab, *Chionoecetes opilio*. The fungi *Phoma* sp. gained prominence with the discovery of phomactins, which are a novel group of terpenoid platelet activating factor antagonists with potential use in the treatment of inflammatory diseases and organ transplant rejection (Goldring *et al.*, 2006). In culture, *Phoma* sp. has been known to produce a diverse array of secondary metabolites such as the isolation of the antimicrobial compound 2-hydroxy-6-methylbenzoic acid from the endophytic *Phoma* sp. from the plant *Taraxacum mongolicum* (Zhang *et al.*, 2013). Hoffman *et al.* (2008) reported the identification of the chemical compound phomodione with potent antibacterial activities against *S. aureus* from *Phoma* sp. However only three out of the five *Phoma* sp. strains in this study displayed antibacterial activity towards test bacteria and the *Phoma* sp. strain (JQ388280.1) from *I. pes-caprae* was the only one among the rest of its kind to demonstrate activity against *S. aureus*.

The second most common isolated endophytic fungi in this study were *Bipolaris* sp. comprising of almost 11.8% of all isolated endophytic fungi. All the strains were isolated from the plant *I. pes-caprae* with none from *V. rotundifolia*. Out of the four *Bipolaris* sp. strains, two of them exhibited antibacterial activity with one strain with promising and wide spectrum activity against 85% of test bacteria in this study. The genus *Bipolaris* has been notoriously known as a plant pathogen due to their ability to produce mycotoxins and phytotoxins that in various instances are detrimental towards plants. Although mostly known for their role as plant pathogens, *Bipolaris* sp. has also been reported to produce biologically active metabolites as in studies by Arunpanichlert

et al. (2012), whereby a dimeric chromanone and a phthalide with antimicrobial and antioxidant activities was isolated from a strains of *Bipolaris* sp. derived from the seagrass, *Halophila ovalis*. While the *Bipolaris* sp. strain in this present study displayed broad spectrum antibacterial activity against both Gram positive and negative bacteria, the strains used in the study by Arunpanichlert *et al.* (2012) only managed to exhibit activity against the Gram positive bacteria *S. aureus*. These findings possess certain similarities to the present study whereby the *Bipolaris* sp. with promising antibacterial activities in this study was also derived from a marine associated plant. This also serves to prove that although the fungi *Bipolaris* sp. may not be exclusive to the terrestrial habitat and may be found in the marine environment as well.

The endophytic fungi *Myrothecium* sp. in this study displayed promising antibacterial activities against Gram positive bacteria. This fungus was previously reported to possess antifungal activities which led to the isolation of antifungal trichothecenes (Liu *et al.*, 2005). A study by Pervez *et al.* (2012) found the fungi *Myrothecium* sp. to possess good in-vitro antimicrobial activities against human pathogens such as *Salmonella typhi*, *Klebsiella pneumonia*, *B. cereus*, including the Gram negative *E.coli* while in comparison, the same fungi strain in the present study was solely potent against Gram positive bacteria. Research by Garcia *et al.* (2012) reported the display of broad spectrum of antibacterial activity from the endophytic fungi *Cochliobolus* sp. isolated from the plant *Sapindus saponaria* L. However in the present study the *Cochliobolus eragrostidis* strain displayed a more narrow range of antibacterial activity targeted more towards Gram positive bacteria while *Cochliobolus lunatus* exhibited no activity. One of the 2 *Dothideomycete* sp. isolated from *I.pes-caprae* demonstrated potent antibacterial activity even managing to inhibit the bacterium *P. aeruginosa*. Previous studies by Rhoden *et al.* (2012) have also reported

antibacterial activities by *Dothideomycete* sp.; the difference being that the strain used in their study was isolated from a terrestrial medicinal plant.

Some of the other strains with a wide range of antibacterial activity covering at least 3 test bacteria were *Paecilomyces* sp., *Montagnulaceae* sp., *Fusarium equiseti*, *Minimidochium* sp., *Penicillium* sp. and *Curvularia* sp. *Curvularia* sp. have been known to produce haloperoxidase which is an enzyme with antimicrobial properties and has also been considered as a potential candidate for the development of surface disinfectants (Hansen *et al.,* 2003). Furthermore there have also been reports on antifungal activities from *Curvularia* sp. (Zhang *et al.,* 2011). There have been no reports on the antibacterial or antimicrobial activities from the fungi *Montagnulaceae* sp. and *Minimidochium* sp. Some of the endophytic fungi strains with reported antibacterial activity in previous studies have displayed no such activities in the present study. For instance the strain *Clasdosporium cladosporioides* in the present study exhibited no antibacterial activity towards any of the test bacteria but a study by Wang *et al.* (2007) stated the isolation of the antimicrobial compound brefeldin A from this genus. Another similar study by Ding *et al.* (2008) also reported the isolation of antimicrobial metabolites with antibacterial activities against Gram positive and negative bacteria from *Clasdosporium* sp. that was found to be associated with the red algae *Porphyra yezoensis*. Another instance where this fungus was isolated from the marine habitat is as reported in Qi *et al.* (2009) in which *Clasdosporium* sp. was isolated from mangrove seawater and was reported to possess antibacterial activities against fouling bacteria. There have been several reports where the fungi *Clasdosporium* sp. has been isolated as endophytic fungi especially from medicinal plants such as studies by, Powthong *et al.* (2012), Vaz *et al.* (2012) and Wang *et al.* (2007) which is similar to the present study as the *C. cladosporioides* strain obtained was from the plant *I.pes-caprae* which also is known for its medicinal values as elaborated earlier. *Fusarium equiseti* is

a fungus usually reported from the terrestrial environment however Llamas *et al.* (2008) reported the isolation of this fungal strain from fluvial channels and sea beds from the south eastern coast of Spain. Though there has been no report of bioactivity from marine derived *F. equiseti*, Shiono *et al.* (2013) stated the isolation of the antimicrobial compound fusaequisin from an endophytic fungi *F. equiseti* that was isolated from a medicinal plant. Furthermore there have also been reports of cytotoxic compound isolated from *F. equiseti* (Morrison *et al.*, 2001).

5.4 Manglicolous fungi as promising natural product reservoirs

Around 90.91% of the manglicolous fungi in this study exhibited antibacterial activity against at least one test bacteria with the highest activity recorded against the Gram positive bacteria, *B. subtilis*, *S. aureus* and *B. cereus* at 90.91%, 81.82% and 72.73% respectively. This selective antibacterial activity displayed by the manglicolous fungi against Gram positive bacteria is similar to studies by Buatong *et al.* (2011) which reported high activity by manglicolous fungi towards the Gram positive *S.aureus* but minimal activity and lack of activity against the Gram negative *E. coli* and *P. aeruginosa* respectively.

The manglicolous fungi displayed no antibacterial activity against *E. faecalis* and *P. aeruginosa* while the lowest percentage was recorded against *E. coli* at 18.18%. Studies have shown that Gram negative bacteria are more resistant to antimicrobials due to the presence of the outer membrane permeability barrier which acts as a barrier to limit the influx of antimicrobials to their targets in the bacterial cell (Poole, 2001). Furthermore, Gram negative bacteria belonging to the Enterobacteriaceae family (eg: *E. coli*) have been proven as serious threats in nosocomial infections due to their spread of resistance (Paterson, 2006). Bacteria from the Enterobacteriaceae family have been reported to produce enzymes such as Extended Spectrum Beta Lactamase (ESBL) which are able to break down many commonly used antibiotics thus providing a probable justification to the lack of activity exhibited by the fungal extracts towards *E. coli*.

Many marine derived fungi have been reported to be able to produce secondary metabolites with interesting bioactivities, some with selective activity and other with broad spectrum of activities. Studies have reported fungi such as *Verruculina enalia* and *Corollospora maritima* with the potential to produce antibacterial compounds. Liberra

et al. (1998) reported the isolation of a novel antibacterial agent, corollosporine from the marine derived fungi *C. maritima*. Corollosporine is an antibiotic with phthalide activity against *S. aureus* and *B. cereus* (Neumann *et al.*, 2006). Research by Lin *et al.* (2002) isolated the metabolites enalin A and B from the mangrove derived fungi *V.enalia* which was isolated from salt lake in the Bahamas. Therefore the antibacterial activities displayed by the fungi *V. enalia* and *C. maritim*a in this study, could have been due to the presence of the reported antibacterial compounds known to be produced by these strains.

Li *et al.* (2010) claimed that the mangrove on the South China Sea coast plays host to an abundant diversity of marine derived fungi that may be an important source of new and novel metabolites with interesting bioactivities. Recently, the research of natural products from marine derived fungi experienced a boost when a group of Chinese researchers' uncovered the immensely large fungal diversity in the unique mangrove habitats on the tropical island of Hainan (Ebel, 2012). Ebel, (2012) stated that only 6% of all new compounds from marine derived fungi originated from mangrove derived fungi. This suggests that though the fungal diversity in mangrove habitats is vast, they have yet to be fully explored for their bioactive metabolites.

5.5 Effect of culture conditions in the production of fungal secondary metabolites

Culture conditions of fungi have been proven to modulate the types and physiochemical properties of secondary metabolites produced, thus altering the levels and intensity of their bioactivity as stated in studies by Ramos *et al.* (2011), Sood, (2011) and Kang *et al.* (2013). A study by Ramos *et al.* (2011) reported differential bioactivity displayed by the same fungal strain with differing incubation times and carbon and nitrogen sources.

In the present study, the antibacterial activities of all marine derived fungi were firstly evaluated using the plug assay which did not require the extraction of secondary metabolites. Two different growth media namely; PDA and CMA was used. Based on optimization testing, the fungi grown on PDA demonstrated better antibacterial activity compared to the same fungi grown on CMA. In accordance to Moss, (1984) and Jennings, (1995), PDA has been characterized as a simple media suitable for the production of fungal secondary metabolites. Several studies on bioactive fungal secondary metabolites have also reported the use of PDA as their primary fungal media (Guo *et al.*, 2006; Varughese *et al.*, 2012). Therefore the types of media used can influence the bioactivity exhibited by the fungi in this study.

According to Bhadury *et al.* (2006), the syntheses of most fungal secondary metabolites are modulated by the polyketide synthase (PKS) and nonribosomal peptide synthethase (NRPS) pathways. Research has shown that the manipulation in fermentation and post genomic technologies may play a prominent role in the expression of these gene clusters thereby influencing the amount and types of secondary metabolites produced by the fungi (Bhadury *et al.*, 2006). These reports serve to prove that the difference in culture conditions determines the secondary metabolites produced by a fungus.

The culture conditions of the manglicolous fungi were manipulated in terms of, incubation time and shaken or stationary phase for optimization purposes. In this present study, incubation times of 10, 15 and 20 days were experimented for optimization. Based on findings, fungi cultured with an incubation period of 10 days or less displayed no antibacterial activity while an incubation period of 20 days showed neither significant increase nor decrease in antibacterial activity displayed. The results of this optimization are not shown in this write up. Deduke *et al.* (2012) stated that due to the fact that fungal secondary metabolites are not required in primary metabolism, their synthesis may be triggered when the nutrients become limited and growth slows down. After optimization of incubation timing, fungi mycelia and fermentation broth were harvested after 15 days of incubation for secondary metabolite extraction.

In a bid to mimic the physiochemical characteristics of their natural environment, all manglicolous fungi in the present study were grown in broth supplemented with salt at a concentration of 25 parts per million (ppm) similar to the stated salinity of the surroundings from which they were isolated, and incubated under agitation phase. Based on experimental optimization, the ideal culture phase for these fungi was found to be the agitation phase of 100 rpm. A recently report by Kusari *et al.* (2012) stated that altering fermentation parameters such as media type, aeration, temperature, pH, agitation and harvest points may influence the production of biologically active metabolites in fungi. Thereby this may serve as a justification for optimal antibacterial activity display of the fungi when cultured at a salt concentration of 25 ppm and the agitation phase of 100 rpm.

5.6 Antibacterial potential of *Saccardoella rhizophorae*

A strain of *S. rhizophorae* was selected from the panel of manglicolous fungi strains used in this study for further chemical analysis of its secondary metabolites. This selection was based on the fact that the crude extract of this fungus displayed activity against five out of the seven test bacteria with promising activity against the Gram negative *E. coli* at a MIC of 500µg/ml. Furthermore all the MIC values recorded by the crude extract of *S. rhizophorae* were 500µg/ml and below suggesting promising antibacterial potential.

Mangrove derived fungi has been a focal point of study since a group of Chinese researchers' isolated numerous significant and new metabolites from this group of fungi (Huang *et al.*, 2008). Li *et al.* (2010) stated that the importance of the mangrove forest on the South China Sea for the discovery of new bioactive chemical compounds as many novel compounds have been reported from this area. The fungi *S. rhizophorae* also originates from the mangrove bordering the South China Sea.

The strain *S. rhizophorae* was isolated from the driftwood of the mangrove tree *Rhizophora apiculata*. Previous studies by Sahoo *et al.* (2009) stated the isolation of the fungi *Clasdosporium herbarum*, *Fusarium moniliforme*, *Cirrenalia basiminuta* and *Halophytophthora vesicular* from the dead leaves of *R.apiculata* with pectinolytic, proteolytic and amylolytic activities. Rukachaisirikil *et al.* (2012) isolated endophytic fungi from *R. apiculata* with antibacterial and antifungal activity. Based on these reports, there may be promising pharmacological potential yet to be fully explored in the fungi found to be associated with *R. apiculata* and that is what this study intends to uncover. Mangrove plants are constantly subjected to the harsh surroundings of the mangrove forest such as high salinity and tidal flushes and thus these plants have been known to have developed special morphological and physiological characteristics as an

adaptation strategy (Wu *et al.*, 2008). Therefore the fungi found to be associated with these plants are also subjected to such harsh and extreme conditions thereby generating the possibility that these fungi may possess the capability to generate certain survival strategies such as secondary metabolites.

In this study we employed small scale cultivation of fungi to obtain their secondary metabolites which led to the isolation of two compounds mainly comprising of fatty acids with antibacterial activities. Numerous studies such as Lin *et al.* (2008), Klaiklay *et al.* (2012), Rukachaisirikul *et al.* (2012) and Amrani *et al.* (2012) have reported the isolation of more than at least five compounds from a single mangrove derived fungi. Therefore the cultivation method used in this study could have led to the small scale of discovery with respect to this study.

According to Bhadury *et al.* (2006), large scale cultivation of marine fungi using bioreactor technologies is crucial to ensure optimal production of important fungal secondary metabolites. The maintenance of standard culture parameters during the entire course of cultivation ensures optimal production of secondary metabolites in terms of quantity and quality. Since the cultivation of fungi in this study was carried out in lab conditions, there could have been slight differences in culture conditions from time to time therefore leading to the discovery of only two major metabolites from *S. rhizophorae*.

The efficacy of antibiotics against bacteria primarily depends on the antimicrobial susceptibility of the bacteria in concern. The MIC values of antibiotics penicillin G and chloramphenicol against test bacteria tabulated against antimicrobial susceptibility interpretive criteria as obtained from The European Committee on Antimicrobial Susceptibility Testing (EUCAST), 2013, is showed in Table 5.1. Based on MIC values of penicillin G, all test bacteria except *E.coli* can be characterized as

resistant strains while based on the MIC values of chloramphenicol, *E. coli* is a susceptible strain, *P. aeruginosa* and *E. faecalis* are resistant strains while *S. aureus, B. cereus, B. subtilis* and *M. luteus* are intermediate strains. The resistant trait displayed by *P. aeruginosa* and *E. faecalis* may justify the lack of activity exhibited of the pure compounds towards this two bacterial strains. Both linoleic and palmitic acid did not exhibite antibacterial activity towards *E. faecalis* which can be justified due to resistant trait displayed by this bacterium against both chloramphenicol and penicillin G; however the other four Gram positive bacterial strains are characterized as intermediate strains according to the MIC values of chloramphenicol thus rendering them more susceptible to the antibacterial action of the pure compounds.

Table 5.1: Comparison of MIC values of antibiotics against test bacteria towards antimicrobial susceptibility interpretive criteria (adapted from EUCAST, 2013)

Bacteria	**Antibiotic**	**MIC of Test bacteria (µg/ml)**	**Antimicrobial susceptibility interpretive criteria (values in µg/ml)**		
			Susceptible	**Intermediate**	**Resistant**
S. aureus	Penicillin	3.91	≤0.12		≥0.25
	Chloramphenicol	31.25	≤8	16	≥32
E. coli	Penicillin	125	≤16	32-64	≥128
	Chloramphenicol	7.81	≤8	16	≥32
P. aeruginosa	Penicillin	≥250	≤64		≥128
	Chloramphenicol	125	≤8	16	≥32
E. faecalis	Penicillin	≥250	≤8		≥16
	Chloramphenicol	≥250	≤8	16	≥32
B. cereus	Penicillin	62.5	≤8		≥16
	Chloramphenicol	15.63	≤8	16	≥32
B. subtilis	Penicillin	62.5	≤8		≥16
	Chloramphenicol	15.63	≤8	16	≥32
M. luteus	Penicillin	125	≤8		≥16
	Chloramphenicol	15.63	≤8	16	≥32

5.6.1 Bioactive metabolites of Saccardoella rhizophorae

Most studies on the identification of fungal metabolites such as Huang *et al.* (2006), Isaka *et al.* (2009) and Klaiklay *et al.* (2012), report the use of column chromatography to fractionate the crude fungal extract. However in the present study, solvent partitioning was used to further fractionate the EA crude fungal extract of *S. rhizophorae* (EAS) prior to column chromatography to ease the separation and isolation of the active. Since EA generally extracts out most of the secondary metabolites from the fermentation broth of the fungi, solvents of differing polarities were used to perform a preliminary separation of secondary metabolites based on polarity. Results obtained showed that out of the three fractions, the *n*-hexane (HS) and dichloromethane (DS) fractions exhibited antibacterial activity while the methanol (MS) fraction did not exhibit any antibacterial activity. This concludes that the active compounds in the fungal extract are of lesser polarity thus they are able to dissolve in solvents with lower polarities as the solvents *n*-hexane and dichloromethane are lower in polarity in comparison to the relatively polar solvent methanol.

Based on the display of antibacterial activities, sub-fractions 2 and 3 of the HS fraction were further subjected to separation via multiple TLC profiling which led to the isolation two fatty acids, both eluted using solvent system, *n*-hexane: ethyl acetate, 75:25. The fatty acid *cis*-9, *cis*-12-octadecadenoic acid (linoleic acid), a polyunsaturated fatty acid was isolated from sub-fraction 2 while the other fatty acid, *n*-hexadecanoic acid (palmitic acid) was isolated from sub-fraction 3. Both the fatty acids isolated are long chain fatty acids which are rather non-polar in nature thus justifying why they were isolated from the non-polar HS fraction.

Linoleic acid exhibited antibacterial activity towards *S. aureus* and *B. subtilis* while the sub-fraction from which linoleic acid was derived from exhibited antibacterial

activity against *B. cereus*, *E. faecalis* and *M. luteus*. The exhibition of antibacterial activity by linoleic acid is lower than its unpurified subfraction which could be due to the synergistic effect (Pereira *et al*., 2014) between linoleic acid and the elements in the unpurified subfraction, thereby increasing the antibacterial effect of the subfraction. This may explain the higher potency of the subfraction in comparision to the pure compound. Palmitic acid exhibited antibacterial activity against *S. aureus, B. cereus, B. subtilis* and *M. luteus* while the subfraction from which palmitic acid was derived from exhibited antibacterial activity only against *S. aureus, B. cereus, B. subtilis*. The exhibition of antibacterial activity by palmitic acid was greater than its subfraction which could be due to the antagonistic effect (Oshiro *et al*., 2008) between palmitic acid and the elements in the unpurified subfraction thereby increasing the antibacterial potency of the purified palmitic acid. This may explain the higher potency of the pure compound in comparison to the unpurified subfraction.

Palmitic acid exhibited antibacterial activity against most of the Gram positive bacterial strains in this study namely; *S. aureus*, *B. cereus, B. subtilis* and *M. luteus*; while linoleic acid exhibited antibacterial activity only against *S. aureus* and *B. subtilis*. The lipo-polysaccharide (LPS) layer of Gram negative bacteria prevents the penetration of hydrophobic oils and their accumulation in the bacterial cell membrane (Bajpai *et al*., 2013). Thus, since fatty acids are known to be hydrophobic in nature, the selective permeability of the LPS layer can justify the lack of antibacterial activity exhibited by both fatty acids against the Gram negative bacteria used in the present study.

According to Zheng *et al.* (2005), fatty acids have been long used as food additives to prevent and inhibit the growth of microorganisms. Certain fatty acids have been known to display antibacterial activities such as in studies by Huang *et al.* (2011), which reported the antibacterial activity of palmitic acid against oral microorganisms. Even the fatty acid linoleic acid has been reported to possess antibacterial activities

against *S. aureus* in studies by Dilika *et al.* (2000) which is similar to the present study in which linoleic acid displayed an MIC of 0.5mg/ml against *S. aureus*. Furthermore, linoleic acid was found to be able to inhibit the bacterial enoyl-acyl carrier protein reductase which is an important component in bacterial fatty acid synthesis thus also inhibiting the growth of the bacteria. Besides being known for its antibacterial activity, fatty acids have also been explored in the field of cancer drug discovery. A study on metabolites from the marine fungi *Trichoderma* sp. by Kandasamy *et al.* (2012) reported the isolation of linoleic acid which was found to be an inhibitor against skin cancer protein thus providing promising potential for future cancer drug development. Furthermore, linoleic acid is polyunsaturated fatty acids (PUFA) which is an essential lipid in human health and nutrition. Fish oils are the main marine source of PUFA, however in a bid to reduce the cost of production, microorganisms are now being explored as sources for the alternative production of PUFA to (Ruiz *et al.*, 2007). The study by Ruiz *et al.* (2007) is similar to the present study which saw the isolation of linoleic acid from the marine derived fungi *Trichoderma longibrachiatum*.

Linoleic acid and palmitic acid are also known to be common fatty acid constituents in essential oils (Tang *et al.*, 2013). Linoleic acid especially is the second major fatty acid after oleic acid in argan oil (Benzaria *et al.*, 2006). The composition of 44.8% oleic acid and 33.7% of linoleic acid in argan oil has been proven to possess nutritional benefits in the reduction of atherosclerosis consequently preventing occurances of cardiovascular diseases in humans (Cherki *et al.*, 2006). Studies by Cherki *et al.* (2006) have stated that linoleic acid, an essential acid that cannot be synthesized by the human body, is a precursor for the synthesis of arachidonic acid which is a precursor of prostaglandin E1, prostacyclin and thromboxane; all of which are known to possess platelet anti-aggregator and vasodilator activities thereby justifying the anti-hypertensive effect of linoleic acid. Arachidonic acid by itself is

known for its hypocholecterolemic effect thus being the reason why oils rich in arachidonic acid derived fatty acids particularly linoleic acid are recommended to reduced total cholesterol (Cherki *et al.*, 2006). Linoleic acid and palmitic acid are also the second major fatty acid component after oleic acid in olive oil with linoleic acid at 5.3-21.0% and palmitic acid at 7.5-20.0% (Mendoza *et al.*, 2013). Olive oil is one of the most commonly consumed cooking oils in Mediterranean countries mainly due to its health benefits especially in prevention of cardiovascular diseases, a similar trait observed in argan oil (Maggio *et al.*, 2009). Palmitic acid, being a saturated fatty acid holds less nutritional values in comparison to the unsaturated omega-6 linoleic acid thus justifying as to why food oils higher in linoleic acid is commonly proven beneficial (Benzaria *et al.*, 2006).

The demand for fatty acid methyl esters (FAMEs) as diesel fuel (biodiesel) is on the rise due to the instability of petroleum prices (Vicente *et al.*, 2009). Biodiesel is fast becoming the favoured alternative choice of fuel in comparison with conventional diesel fuels especially in countries such as European Union, America, Australia, Japan, China, and Malaysia among others (Zhu *et al.*, 2008). Fatty acids are known to be ubiquitously found in most living organisms such as animals and plants. Although vegetable oils and animal fats are the main sources of fatty acids but their sustainability is mostly environmental dependant and varies with climates and seasons, thus forcing scientist to explore alternative sources of fatty acids in order to meet the demands of the biodiesel industry (Zhu *et al.*, 2008; Khot *et al.*, 2012). Currently lipids from oleaginous microorganisms may prove to possess significant potential as feedstock for biodiesel production as its resource requirements are basic and therefore may prove economical (Zhu *et al.*, 2008).. Microalgae have been known to be the most prolific source for fatty acids among all microorganisms (Dominguez *et al.*, 2012).

The production of fatty acids by fungi and its application have been studied since the late nineteenth century and fungi have also been known to be good producers of commercially important fatty acids (De *et al.*, 2011). For instance, the fungus *Mucor circinelloides* produces large quantities of fatty acids thus making it a suitable candidate for large scale commercial production (Vicente *et al.*, 2009). Although there have been not as many reports of marine derived fungi as potential producers of fatt acids, Table 5.2 shows several selected marine derived fungi strains reported as significant producers of the same fatty acids as reported in the present study.

Table 5.2: Research done on selected marine derived fungi leading to the isolation of similar fatty acids as in the present study

Fungi	**Fatty acids**	**Reference**
Trichoderma sp.	Linoleic acid	Kandasamy *et al.* (2012)
Orpinomyces sp.	Palmitic acid	Comlekcioglu *et al.* (2010)
Paecilomyces sp.	Linoleic acid	Wang *et al.* (2010)
Mortierella alpina	Linoleic acid	Sakuradani *et al* (2009)
Ceriporiopsis subvermispora	Linoleic & palmitic acid	Enoki *et al.* (1999)
Hypoxylon sp.	Linoleic acid	Le *et al.* (1999)
Arthrobotrys conoides	Linoleic acid	Anke *et al.* (1995)
Annulohypoxylon squamulosum	Palmitic acid	Cheng *et al.* (2012)
Alternaria sp. M6	Palmitic acid	Zhang *et al.* (2012)
Gliomastix murorum & *Pichia guilliermondii*	Palmitic acid	Zhao *et al.* (2009)
Chaetomium fusiforme	Palmitic acid	Guo *et al.* (2008)
Cunninghamella echinulata	Linoleic acid	Fakas *et al.* (2008)
Leccinum extremiorientale	Palmitic acid	Gao *et al.* (2003)
Julella avicenniae	Palmitic acid	Le *et al.* (1998)
Clitocybe laccata, Lactarius controversus, Phellinus pomaceus, Stereum hirsutum	Palmitic & linoleic acid	Senatore *et al.* (1988)

5.7 Improvisations with respect to the present study and suggestions for future work

- The endophytic fungi strains in the present study were identified based on the sequence of only one gene (ITS) whereby some fungal strains were only identified to genus level. Therefore future work should adopt multi-gene analysis to improvise the accuracy in identifying the endophytic fungi and also to conduct phylogenetic studies of endophytic fungi found in the two marine associated plants in the study.
- The present study focused on endophytic fungi that were able to grow in given laboratory conditions. Proposed future work should focus also on the non-culturable endophytic fungi strains from the given plants.
- The present study only involved the chemical analysis of *S. rhizophorae* secondary metabolites. Therefore future work should focus on the other fungi strains especially the endophytic fungi strains that displayed promising antibacterial activities in the broth microdilution assay.
- Studies by Ramos *et al.* (2011), Sood, (2011) and Kang *et al.* (2013) have reported differing production of secondary metabolites with respect to varying culture conditions. In the present study, culture conditions were manipulated only in accordance to basic experimental optimization. Therefore further studies should involve systematic manipulation of culture conditions to obtain standard curves of varying culture conditions in relation to secondary metabolite profiles.
- In the bioassay guided fractionation, only the HS fraction was purified which led to the isolation the linoleic acid and palmitic acid. The DS fraction exhibited significant antibacterial activity mainly against the Gram positive bacteria strains. However the dichloromethane fraction could not be further purified due to small amount of the fraction. Thus future work should focus on the large scale

cultivation and extraction of fungal crude extract to ensure complete isolation and analysis of their secondary metabolite profiles including minor compounds.

- This is a bioassay-guided fractionation study therefore the inactive fractions of the *S. rhizophorae* extract were not purified. Thus future studies should also focus on inactive fractions and sub-fractions; this is because although the fractions and sub-fractions may be inactive, there may be metabolites present that may possess activity in its purified form.

CHAPTER 6

CONCLUSIONS

The diversity of endophytic fungi in the marine associated plants *Ipomoea pes-caprae* and *Vitex rotundifolia* is noteworthy. Twenty-one endophytic fungi strains were isolated from *I. pes-caprae* comprising of fifteen genus/species. As for *V. rotundifolia*, thirteen endophytic fungi strains were isolated and identified comprising of eight genus or species. Certain endophytic fungi such as *Bipolaris* sp., *Guignardia mangiferae* and *Phoma* sp., are known plant pathogens thus suggesting that they may not be exclusively pathogenic but may have evolved into a symbiotic relationship with the plant. Endophytic fungi have long been associated with the production of pharmacologically active metabolites which is supported by the findings of this study. Ten and five of the endophytic fungi from *I. pes-caprae* and *V. rotundifolia* respectively exhibited antibacterial activity against the test bacteria in the study. However the majority of the endophytic fungi strains were active against Gram positive bacteria with only 11.76% of all fungi strains active against Gram negative bacteria. As suggested by hypothesis on the influence of the place of collection and ethobotanical significance of a plant on the bioactivities exhibited by their endophytic fungi, the antibacterial activities displayed by the fungi strains from *I. pes-caprae* were broader spectrum. A total of five endophytic fungi were chosen for further analysis through the broth microdilution assay and strains such as *Bipolaris* sp. (HQ631009.1) from *I. pes-caprae* and *Paecilomyces* sp. from *V. rotundifolia* exhibited the strongest antibacterial potential with the lowest MIC values.

The manglicolous fungi in the study exhibited moderate antibacterial activities overall with the majority of them active against the Gram positive test bacteria; a trait common to that displayed by the endophytic fungi in the present study. Out of the eleven manglicolous fungi, ten or 90.9% of them exhibited antibacterial

activity. However out of this 90.9%, only 20% were active against Gram negative bacteria. The results obtained from the preliminary plug assay were in sync with those obtained from the broth microdilution assay.

The chemical elucidation of metabolites from the crude extract of *Saccardoella rhizophorae* revealed the presence of fatty acids linoleic acid and palmitic acid. These fatty acids although considered ubiquitious and simple, may prove beneficial as important constituents of essential oils and also possesses nutritional values as suggested by the role of linoleic acid in particular. Besides this, the fungal strain *S. rhizophorae* may prove to be a potential candidate to be explored for its role in the sustainable production of raw materials for biodiesel fuel.

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APPENDIX 1

Preparation of media and chemicals

Luria Broth agar (LB)

37 g of powder was dissolved with 1 L distilled water in a Schott bottle. The medium was then sterilized by autoclave at 121°C for 15 minutes.

Mueller-Hinton broth (MHB)

21 g of powder was dissolved with 1 L distilled water in a Schott bottle. The medium was then sterilized by autoclave at 121°C for 15 minutes.

Potato Dextrose agar (PDA)

39 g of powder was dissolved in 1 L distilled water in a Schott bottle. The medium was then sterilized by autoclave at 121°C for 15 minutes.

Potato Dextrose broth (PDB)

24 g of powder was dissolved in 1 L distilled water in a Schott bottle. The medium was then sterilized by autoclave at 121°C for 15 minutes.

Preparation of antibiotics

For the preparation of antibiotics chloramphenicol and penicillin, 2.0 mg of powder was dissolved in 1ml of distilled water respective for both antibiotics. It was then sterilized using 0.45 μm syringe filter. After sterilization, the antibiotic solution was stored in a -20°C freezer.

Preparation of INT

20 mg of INT was dissolved in 50 ml of distilled water. It was then sterilized using 0.45 μm syringe filter. After sterilization, the INT solution was stored in a -20°C freezer.

APPENDIX 2

List of aligned ITS sequence by NCBI BLAST of marine derived endophytic fungi

Bipolaris sp. KH00278 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

Sequence ID: gi|294471397|gb|GU017499.1|Length: 577Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1066 bits(577)	0.0	577/577(100%)	0/577(0%)	Plus/Plus

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Query  19   AACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAATACAATATGAAGGCTG  78
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    AACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAATACAATATGAAGGCTG  60
Query  79   TCCGCAGCTGGAGTATTTTATTACCCTTGTCTTTTGCGCACTTGTTGTTTCCTGGGCGGG  138
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TCCGCAGCTGGAGTATTTTATTACCCTTGTCTTTTGCGCACTTGTTGTTTCCTGGGCGGG  120
Query  139  TTCGCTCGCCACCAGGACCACCAAATAAACCTTTTTTATGCAGTTGCAATCAGCGTCAGT  198
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TTCGCTCGCCACCAGGACCACCAAATAAACCTTTTTTATGCAGTTGCAATCAGCGTCAGT  180
Query  199  ACAAACAATGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG  258
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  ACAAACAATGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG  240
Query  259  AAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATC  318
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATC  300
Query  319  TTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTG  378
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  TTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTG  360
Query  379  TACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTTGTCTTTGGTCGCCCAAAGACTCGCCT  438
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTTGTCTTTGGTCGCCCAAAGACTCGCCT  420
Query  439  TAAAGTGATTGGCAGCCGGCCTTTCTGGTTTCGCAGCGCAGCACATTTTTGCGCTTGCCA  498
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  TAAAGTGATTGGCAGCCGGCCTTTCTGGTTTCGCAGCGCAGCACATTTTTGCGCTTGCCA  480
Query  499  TCAGCAAAACGGCAATCCATCAAGCCTCCTTCTCACGTTTGACCTCGGATCAGGTAGGGA  558
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  TCAGCAAAACGGCAATCCATCAAGCCTCCTTCTCACGTTTGACCTCGGATCAGGTAGGGA  540
Query  559  TACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA                        595
            |||||||||||||||||||||||||||||||||||||
Sbjct  541  TACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA                        577
```

Bipolaris sp. 1 TMS-2011 voucher SC9d100p9-2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: gi|317383330|gb|HQ631009.1|Length: 609Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1068 bits(578)	0.0	589/594(99%)	2/594(0%)	Plus/Plus

```
Query  2    GGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAAT  61
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16   GGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAAT  75
Query  62   ACAATATGAAGGCTGTCCGCAGCTGGAGTATTTTATTACCCTTGTCTTTTGCGCACTTG-  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| 
Sbjct  76   ACAATATGAAGGCTGTCCGCAGCTGGAGTATTTTATTACCCTTGTCTTTTGCGCACTTGA  135
Query  121  TTGTTTCCTGGGCGGGTTCGCTCGCCACCAGGACCACCAAACAAACCTTTTTTATGCAGT  180
            |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  136  TTGTTTCCTGGGCGGGTTCGCTCGCCACCAGGACCACCAAATAAACCTTTTTTATGCAGT  195
Query  181  TGCAATCAGCGTCAGTACAAACAATGTAAATCATTTACAACTTTCAACAACGGATCTCTT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  196  TGCAATCAGCGTCAGTACAAACAATGTAAATCATTTACAACTTTCAACAACGGATCTCTT  255
Query  241  GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  256  GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTC  315
Query  301  AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  316  AGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCT  375
Query  361  GTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTTGTCTTTGGTC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  376  GTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTTGTCTTTGGTC  435
Query  421  GCCCAAAGACTCGCCTTAAAGTGATTGGCAGCCGGCCTTTCTGGTTTCGCAGCGCAGCAC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  436  GCCCAAAGACTCGCCTTAAAGTGATTGGCAGCCGGCCTTTCTGGTTTCGCAGCGCAGCAC  495
Query  481  ATTTTTGCGCTTGCCATCAGCAAAACGGCAATCCATCAAGCCTCCTTCTCACGTTTGACC  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  496  ATTTTTGCGCTTGCCATCAGCAAAACGGCAATCCATCAAGCCTCCTTCTCACGTTTGACC  555
Query  541  TCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAG-CGGAGGAA        593
            |||||||||||||||||||||||||||||||||||||||||||  ||||| ||||||||
Sbjct  556  TCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATATATAAGGCGGAGGAA        609
```

Bipolaris sp. CC06-23-3_DCPA 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|478432585|gb|JX960589.1|Length: 570Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1037 bits(561)	0.0	568/571(99%)	2/571(0%)	Plus/Plus

```
Query  15   TCGTAACAAGGTCTCCGTTAAGGTGAACCTGCGGAGGGATCATTACACAATACAATATGA  74
            ||||||||||||||||||  ||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TCGTAACAAGGTCTCCGT--AGGTGAACCTGCGGAGGGATCATTACACAATACAATATGA  58
Query  75   AGGCTGTCCGCAGCTGGAGTATTTTATTACCCTTGTCTTTTGCGCACTTGTTGTTTCCTG  134
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  59   AGGCTGTCCGCAGCTGGAGTATTTTATTACCCTTGTCTTTTGCGCACTTGTTGTTTCCTG  118
Query  135  GGCGGGTTCGCTCGCCACCAGGACCACCAAACAAACCTTTTTTATGCAGTTGCAATCAGC  194
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  119  GGCGGGTTCGCTCGCCACCAGGACCACCAAATAAACCTTTTTTATGCAGTTGCAATCAGC  178
Query  195  GTCAGTACAAACAATGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCA  254
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  179  GTCAGTACAAACAATGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCA  238
Query  255  TCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCAT  314
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  239  TCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCAT  298
Query  315  CGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGT  374
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  299  CGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGT  358
Query  375  CATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTTGTCTTTGGTCGCCCAAAGAC  434
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  359  CATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTTGTCTTTGGTCGCCCAAAGAC  418
Query  435  TCGCCTTAAAGTGATTGGCAGCCGGCCTTTCTGGTTTCGCAGCGCAGCACATTTTTGCGC  494
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  419  TCGCCTTAAAGTGATTGGCAGCCGGCCTTTCTGGTTTCGCAGCGCAGCACATTTTTGCGC  478
Query  495  TTGCCATCAGCAAAACGGCAATCCATCAAGCCTCCTTCTCACGTTTGACCTCGGATCAGG  554
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  479  TTGCCATCAGCAAAACGGCAATCCATCAAGCCTCCTTCTCACGTTTGACCTCGGATCAGG  538
Query  555  TAGGGATACCCGCTGAACTTAAGCATATCAA                               585
            ||||||||||||||||||||||||||||||
Sbjct  539  TAGGGATACCCGCTGAACTTAAGCATATCAA                               569
```

Bipolaris sp. SLP1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence
Sequence ID: gb|JX949177.1|Length: 974Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1799 bits(974)	0.0	974/974(100%)	0/974(0%)	Plus/Plus

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Query  1    GGGGAAAACATTAAATATGAAGGCTGTCCGCAGCTGGAGTATTTTATTACCCTTGTCTTT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GGGGAAAACATTAAATATGAAGGCTGTCCGCAGCTGGAGTATTTTATTACCCTTGTCTTT  60
Query  61   TGCGCACTTGTTGTTTCCTGGGCGGGTTCGCTCGCCACCAGGACCACCAAATAAACCTTT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TGCGCACTTGTTGTTTCCTGGGCGGGTTCGCTCGCCACCAGGACCACCAAATAAACCTTT  120
Query  121  TTTATGCAGTTGCAATCAGCGTCAGTACAAACAATGTAAATCATTTACAACTTTCAACAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TTTATGCAGTTGCAATCAGCGTCAGTACAAACAATGTAAATCATTTACAACTTTCAACAA  180
Query  181  CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAAT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAAT  240
Query  241  TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  TGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAA  300

Query  301  GGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTT  360
Query  361  GTCTTTGGTCGCCCAAAGACTCGCCTTAAAGTGATTGGCAGCCGGCCTTTCTGGTTTCGC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GTCTTTGGTCGCCCAAAGACTCGCCTTAAAGTGATTGGCAGCCGGCCTTTCTGGTTTCGC  420
Query  421  AGCGCAGCACATTTTTGCGCTTGCCATCAGCAAAACGGCAATCCATCAAGCCTCCTTCTC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  AGCGCAGCACATTTTTGCGCTTGCCATCAGCAAAACGGCAATCCATCAAGCCTCCTTCTC  480
Query  481  ACGTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGACGGA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  ACGTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGACGGA  540
Query  541  GGAATACGTCTAGCAACTAACCCAACCCTTCCCTTCTTTCTTCTTTAGATAATAAACGAA  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  GGAATACGTCTAGCAACTAACCCAACCCTTCCCTTCTTTCTTCTTTAGATAATAAACGAA  600
Query  601  ATCAAAGCACACTTGCATTTCCCGAACCACAGTACAGTAGACCATGTATCACTTATTGTC  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  ATCAAAGCACACTTGCATTTCCCGAACCACAGTACAGTAGACCATGTATCACTTATTGTC  660
Query  661  CTGAGTCTTCCGCGCCACGCTACTCGCGAGATAAGACACACTAATCAAATAGAACCTACA  720
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  661  CTGAGTCTTCCGCGCCACGCTACTCGCGAGATAAGACACACTAATCAAATAGAACCTACA  720
Query  721  TCTCAAAAGCTCGCCGACCTCATCAAACTCGCAAAAAGCCAGATTAAAATATGCAACTCA  780
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721  TCTCAAAAGCTCGCCGACCTCATCAAACTCGCAAAAAGCCAGATTAAAATATGCAACTCA  780
Query  781  ACTTCATGAAGTTGGAATCGCTAATAATCGCTGATCATAATGCCACTGCCAAGACGTTCC  840
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781  ACTTCATGAAGTTGGAATCGCTAATAATCGCTGATCATAATGCCACTGCCAAGACGTTCC  840
Query  841  CAGACCTTGTACCACCCCCCGTCACATCATGCGGATTGAGTCGGAGTAGAATTATTTTCC  900
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841  CAGACCTTGTACCACCCCCCGTCACATCATGCGGATTGAGTCGGAGTAGAATTATTTTCC  900
Query  901  CTAACCTTCGGGAAGACGCTTAATATTGATGCATTCTTACTGGGGTACAGTCCACAAATT  960
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901  CTAACCTTCGGGAAGACGCTTAATATTGATGCATTCTTACTGGGGTACAGTCCACAAATT  960
Query  961  GTTTTATTCTACAA                                                 974
            |||||||||||||
Sbjct  961  GTTTTATTCTACAA                                                 974
```

Cladosporium cladosporioides strain F28b 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
Sequence ID: gi|169639302|gb|EU497957.1|Length: 574Number of Matches: 1
Score   Expect  Identities     Gaps    Strand
1061 bits(574) 0.0     574/574(100%)  0/574(0%)      Plus/Plus
Query  1    GGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACAAGTG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GGAAGTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACAAGTG  60
Query  61   ACCCCGGTTTACCACCGGGATGTTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   ACCCCGGTTTACCACCGGGATGTTCATAACCCTTTGTTGTCCGACTCTGTTGCCTCCGGG  120
Query  121  GCGACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GCGACCCTGCCTTCGGGCGGGGGCTCCGGGTGGACACTTCAAACTCTTGCGTAACTTTGC  180
Query  181  AGTCTGAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  AGTCTGAGTAAACTTAATTAATAAATTAAAACTTTTAACAACGGATCTCTTGGTTCTGGC  240
Query  241  ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA  300
Query  301  TCGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTTCGAGCG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  TCGAATCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTTCGAGCG  360
Query  361  TCATTTCACCACTCAAGCCTCGCTTGGTATTGGGCAACGCGGTCCGCCGCGTGCCTCAAA  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TCATTTCACCACTCAAGCCTCGCTTGGTATTGGGCAACGCGGTCCGCCGCGTGCCTCAAA  420
Query  421  TCGACCGGCTGGGTCTTCTGTCCCCTAAGCGTTGTGGAAACTATTCGCTAAAGGGTGCTC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  TCGACCGGCTGGGTCTTCTGTCCCCTAAGCGTTGTGGAAACTATTCGCTAAAGGGTGCTC  480
Query  481  GGGAGGCTACGCCGTAAAACAACCCCATTTCTAAGGTTGACCTCGGATCAGGTAGGGATA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GGGAGGCTACGCCGTAAAACAACCCCATTTCTAAGGTTGACCTCGGATCAGGTAGGGATA  540
Query  541  CCCGCTGAACTTAAGCATATCAATAAGCGGAGGA                             574
            |||||||||||||||||||||||||||||||||
Sbjct  541  CCCGCTGAACTTAAGCATATCAATAAGCGGAGGA                             574
```

Cochliobolus eragrostidis strain NBRC 100188 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

```
Sequence ID: gi|363544572|gb|JN943412.1|Length: 572Number of Matches: 1
Score   Expect  Identities     Gaps    Strand
1057 bits(572) 0.0     572/572(100%)  0/572(0%)      Plus/Plus
Query  27   TCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAAAAAATATGAAGGCTGCAACCGCC  86
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAAAAAATATGAAGGCTGCAACCGCC  60
Query  87   AGTTTTGGCGGGGAAGCTGAATTATTTTTCACCCATGTCTTTTGCGCACTTGTTGTTTCC  146
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   AGTTTTGGCGGGGAAGCTGAATTATTTTTCACCCATGTCTTTTGCGCACTTGTTGTTTCC  120
Query  147  TGGGCGGGTTCGCCCGCCACCAGGACCACACCATAAACCTTTTTTATGCAGTTGCAATCA  206
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TGGGCGGGTTCGCCCGCCACCAGGACCACACCATAAACCTTTTTTATGCAGTTGCAATCA  180
Query  207  GCGTCAGTATAACAAATGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGG  266
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GCGTCAGTATAACAAATGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGG  240
Query  267  CATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATC  326
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATC  300
Query  327  ATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGC  386
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  ATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGC  360
Query  387  GTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTGTCTTTGGCTTTTGCCCCA  446
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTGTCTTTGGCTTTTGCCCCA  420
Query  447  AAGACTCGCCTTAAAACGATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCACATTTTT  506
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  AAGACTCGCCTTAAAACGATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCACATTTTT  480
Query  507  GCGCTTGCAACCAGCTAAAGAGGCCAGCAATCCATCAAGACCTTCTTCTCACTTTTGACC  566
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GCGCTTGCAACCAGCTAAAGAGGCCAGCAATCCATCAAGACCTTCTTCTCACTTTTGACC  540
Query  567  TCGGATCAGGTAGGGATACCCGCTGAACTTAA                              598
            ||||||||||||||||||||||||||||||||
Sbjct  541  TCGGATCAGGTAGGGATACCCGCTGAACTTAA                              572
```

Cochliobolus eragrostidis strain NBRC 32566 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

```
Sequence ID: gi|363544608|gb|JN943448.1|Length: 572Number of Matches: 1
Score   Expect  Identities     Gaps    Strand
1057 bits(572) 0.0    572/572(100%)  0/572(0%)      Plus/Plus
Query  26   TCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAAAAAATATGAAGGCTGCAACCGCC  85
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAAAAAATATGAAGGCTGCAACCGCC  60
Query  86   AGTTTTGGCGGGGAAGCTGAATTATTTTTCACCCATGTCTTTTGCGCACTTGTTGTTTCC  145
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   AGTTTTGGCGGGGAAGCTGAATTATTTTTCACCCATGTCTTTTGCGCACTTGTTGTTTCC  120
Query  146  TGGGCGGGTTCGCCCGCCACCAGGACCACACCATAAACCTTTTTTATGCAGTTGCAATCA  205
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  TGGGCGGGTTCGCCCGCCACCAGGACCACACCATAAACCTTTTTTATGCAGTTGCAATCA  180
Query  206  GCGTCAGTATAACAAATGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGG  265
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GCGTCAGTATAACAAATGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGG  240
Query  266  CATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATC  325
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CATCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATC  300
Query  326  ATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGC  385
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  ATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGC  360
Query  386  GTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTGTCTTTGGCTTTTGCCCCA  445
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTGTCTTTGGCTTTTGCCCCA  420
Query  446  AAGACTCGCCTTAAAACGATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCACATTTTT  505
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  AAGACTCGCCTTAAAACGATTGGCAGCCGGCCTACTGGTTTCGGAGCGCAGCACATTTTT  480
Query  506  GCGCTTGCAACCAGCTAAAGAGGCCAGCAATCCATCAAGACCTTCTTCTCACTTTTGACC  565
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GCGCTTGCAACCAGCTAAAGAGGCCAGCAATCCATCAAGACCTTCTTCTCACTTTTGACC  540
Query  566  TCGGATCAGGTAGGGATACCCGCTGAACTTAA                              597
            ||||||||||||||||||||||||||||||||
Sbjct  541  TCGGATCAGGTAGGGATACCCGCTGAACTTAA                              572
```

Cochliobolus lunatus isolate CATAS-CL01 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
Sequence ID: gi|241912467|gb|GQ169765.1|Length: 562Number of Matches: 1
Score   Expect  Identities     Gaps    Strand
1038 bits(562) 0.0    562/562(100%)  0/562(0%)      Plus/Plus
Query  25   TCCGTAGGTGAACCTGCGGAGGGATCATTACACAAATACAATATGAAGGCTTCGGCTGGA  84
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TCCGTAGGTGAACCTGCGGAGGGATCATTACACAAATACAATATGAAGGCTTCGGCTGGA  60
Query  85   TTATTTATTTCACCCTTGTCTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCCCGCCA  144
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TTATTTATTTCACCCTTGTCTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCCCGCCA  120
Query  145  CCAGGACCACACCATAAACCTTTTTTGTTAATGCAGTCAGCGTCAGTACAACAAATGTAA  204
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CCAGGACCACACCATAAACCTTTTTTGTTAATGCAGTCAGCGTCAGTACAACAAATGTAA  180
Query  205  ATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA  264
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  ATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGA  240
Query  265  AATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT  324
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT  300
Query  325  TGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTT  384
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  TGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTT  360
Query  385  TGCTTGGTGTTGGGCGTTTTGTCTTTGCTCGCCAAAGACTCGCCTTAAAACGATTGGCAG  444
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  TGCTTGGTGTTGGGCGTTTTGTCTTTGCTCGCCAAAGACTCGCCTTAAAACGATTGGCAG  420
Query  445  CCGACCTCTTGGTTTCGCAGCGCAGCACAATTTTGCGCTTGCAAATCAGCAAGTTGGCAC  504
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  CCGACCTCTTGGTTTCGCAGCGCAGCACAATTTTGCGCTTGCAAATCAGCAAGTTGGCAC  480
Query  505  TCCATCAAGAACATTTTCTTACGTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTT  564
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  TCCATCAAGAACATTTTCTTACGTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTT  540
Query  565  AAGCATATCAATAAGCGGAGGA                                        586
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            ||||||||||||||||||||
Sbjct  541  AAGCATATCAATAAGCGGAGGA                                      562
```

Colletotrichum hippeastri strain CBS241.78 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
Sequence ID: gi|399223736|gb|JX010293.1|Length: 612Number of Matches: 1
Score   Expect  Identities     Gaps    Strand
1096 bits(593) 0.0     603/608(99%)   0/608(0%)      Plus/Plus
Query  7    GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGT  66
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  5    GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGT  64
Query  67   TACCGCTCTATAACCCTTTGTGAACATACCTACAACTGTTGCTTCGGCGGGTAGGCCGTC  126
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  65   TACCGCTCTATAACCCTTTGTGAACATACCTACAACTGTTGCTTCGGCGGGTAGGCCGTC  124
Query  127  CCGTGAAAAGGACGCCTCCCGGCCCGGCCCGGACCCCTCGCGGGAACGGACCCGGCGCCC  186
            |||||||||||||||||||||||||||||||||||  ||||| ||||||||||||||||
Sbjct  125  CCGTGAAAAGGACGCCTCCCGGCCCGGCCCGGACCCCCAGCGGGGACGGACCCGGCGCCC  184
Query  187  GCCGGAGGATAACCAAACTCTGTTTTAACGACGTTTCTTCTGAGTGGCATAAGCAAAATA  246
            |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  185  GCCGGAGGATAACCAAACCCTGTTTTAACGACGTTTCTTCTGAGTGGCATAAGCAAAATA  244
Query  247  ATCAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG  306
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  245  ATCAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATG  304
Query  307  CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG  366
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  305  CGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG  364
Query  367  CCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCT  426
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  365  CCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCT  424
Query  427  TGGTGTTGGGGCTCTACGGTTGACGTAGGCCCCCAAAGGTAGTGGCGGACCCTCCCGGAG  486
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  425  TGGTGTTGGGGCTCTACGGTCGACGTAGGCCCCCAAAGGTAGTGGCGGACCCTCCCGGAG  484
Query  487  CCTCCTTTGCGTAGTAACATTTCGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAA  546
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  485  CCTCCTTTGCGTAGTAACATTTCGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAA  544
Query  547  ACCCCCCAATTTTTCAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCA  606
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  545  ACCCCCCAATTTTTCAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCA  604
Query  607  TATCAATA                                                      614
            ||||||||
Sbjct  605  TATCAATA                                                      612
```

Colletotrichum sp. NFCCI 1611 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
Sequence ID: gb|JN390940.1|Length: 595Number of Matches: 1
Score   Expect  Identities     Gaps    Strand
1099 bits(595) 0.0     595/595(100%)  0/595(0%)      Plus/Plus
Query  1    TAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCGCTCTATAAC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACTGAGTTACCGCTCTATAAC  60
Query  61   CCTTTGTGAACATACCTACAACTGTTGCTTCGGCGGGTAGGCCGTCCCCTGAAAAGGACG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CCTTTGTGAACATACCTACAACTGTTGCTTCGGCGGGTAGGCCGTCCCCTGAAAAGGACG  120
Query  121  CCTcccggcccggaccggaccccccgcgggaccggacccggcgcccgccggaGGATAACC  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CCTCCCGGCCCGGACCGGACCCCCCGCGGGACCGGACCCGGCGCCCGCCGGAGGATAACC  180
Query  181  AAACTCTATTGTAACGACGTTTCTTCTGAGTGGCATAAGCAAAATAATCAAAACTTTTAA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  AAACTCTATTGTAACGACGTTTCTTCTGAGTGGCATAAGCAAAATAATCAAAACTTTTAA  240
Query  241  CAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG  300
Query  301  AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCT  360
Query  361  GGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTGTTGGGGCTC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTGTTGGGGCTC  420
Query  421  TACGGTCGACGTAGGCCCTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  TACGGTCGACGTAGGCCCTCAAAGGTAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAG  480
Query  481  TAACATTTCGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCCAATTTTC  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  TAACATTTCGTCTCGCACTGGGATCCGGAGGGACTCTTGCCGTAAAACCCCCCAATTTTC  540
Query  541  CAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATA       595
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  CAAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATA       595
```

Curvularia sp. TMS-2011 voucher SC16d1p11-2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|317383382|gb|HQ631061.1|Length: 629Number of Matches: 1
Score Expect Identities Gaps Strand
1129 bits(611) 0.0 614/615(99%) 1/615(0%) Plus/Plus

```
Query  3    GGAAGTAAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAA  62
            |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16   GGAAGT-AAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAA  74
Query  63   TAAACATATGAAGGCTGCACCGCCAACAGGCGGCAAGGCTGGAGTATTTTATTACCCTTG  122
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  75   TAAACATATGAAGGCTGCACCGCCAACAGGCGGCAAGGCTGGAGTATTTTATTACCCTTG  134
Query  123  TCTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCCCGCCTCCAGGACCACATGATAAA  182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  135  TCTTTTGCGCACTTGTTGTTTCCTGGGCGGGTTCGCCCGCCTCCAGGACCACATGATAAA  194
Query  183  CCTTTTTTATGCAGTTGCAATCAGCGTCAGTACAACAAATGTAAATCATTTACAACTTTC  242
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  195  CCTTTTTTATGCAGTTGCAATCAGCGTCAGTACAACAAATGTAAATCATTTACAACTTTC  254
Query  243  AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAGTG  302
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  255  AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAGTG  314
Query  303  TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATT  362
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  315  TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATT  374
Query  363  CCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCG  422
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  375  CCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCG  434
Query  423  TTTTTTGTCTTTGGTTTTGTCCAAAGACTCGCCTTAAAACGATTGGCAGCCGGCCTACTG  482
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  435  TTTTTTGTCTTTGGTTTTGTCCAAAGACTCGCCTTAAAACGATTGGCAGCCGGCCTACTG  494
Query  483  GTTTCGCAGCGCAGCACATTTTTGCGCTTGCAATCAGCAAAAGAGGACGGCACTCCATCA  542
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  495  GTTTCGCAGCGCAGCACATTTTTGCGCTTGCAATCAGCAAAAGAGGACGGCACTCCATCA  554
Query  543  AGACTCTATATCACTTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATAT  602
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  555  AGACTCTATATCACTTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATAT  614
Query  603  CAATAAGCGGAGGAA                                               617
            |||||||||||||||
Sbjct  615  CAATAAGCGGAGGAA                                               629
```

Curvularia sp. HSAUP074064 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|239586345|gb|GQ184733.1|Length: 589Number of Matches: 1
Score Expect Identities Gaps Strand
1088 bits(589) 0.0 589/589(100%) 0/589(0%) Plus/Plus

```
Query  28   TCCGTAGGTGAACCTGCGGAGGGATCATTACACAATAAACATATGAAGGCTGCACCGCCA  87
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TCCGTAGGTGAACCTGCGGAGGGATCATTACACAATAAACATATGAAGGCTGCACCGCCA  60
Query  88   ACAGGCGGCAAGGCTGGAGTATTTTATTACCCTTGTCTTTTGCGCACTTGTTGTTTCCTG  147
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   ACAGGCGGCAAGGCTGGAGTATTTTATTACCCTTGTCTTTTGCGCACTTGTTGTTTCCTG  120
Query  148  GGCGGGTTCGCCCGCCTCCAGGACCACATGATAAACCTTTTTTATGCAGTTGCAATCAGC  207
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GGCGGGTTCGCCCGCCTCCAGGACCACATGATAAACCTTTTTTATGCAGTTGCAATCAGC  180
Query  208  GTCAGTACAACAAATGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCA  267
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GTCAGTACAACAAATGTAAATCATTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCA  240
Query  268  TCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCAT  327
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  TCGATGAAGAACGCAGCGAAATGCGATACGTAGTGTGAATTGCAGAATTCAGTGAATCAT  300
Query  328  CGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGT  387
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  CGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTCGAGCGT  360
Query  388  CATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTTTGTCTTTGGTTTTGTCCAAA  447
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGCGTTTTTTGTCTTTGGTTTTGTCCAAA  420
Query  448  GACTCGCCTTAAAACGATTGGCAGCCGGCCTACTGGTTTCGCAGCGCAGCACATTTTTGC  507
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GACTCGCCTTAAAACGATTGGCAGCCGGCCTACTGGTTTCGCAGCGCAGCACATTTTTGC  480
Query  508  GCTTGCAATCAGCAAAAGAGGACGGCACTCCATCAAGACTCTATATCACTTTTGACCTCG  567
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GCTTGCAATCAGCAAAAGAGGACGGCACTCCATCAAGACTCTATATCACTTTTGACCTCG  540
Query  568  GATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA             616
```

Dothideomycetes sp. TMS-2011 voucher SC10d50p8-8 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|317383329|gb|HQ631008.1|
Score Expect Identities Gaps Strand

```
896 bits(485)  0.0     581/627(93%)   8/627(1%)      Plus/Plus
Query  4    GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTGTG  63
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  16   GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTTTG  75
Query  64   GCCGCCGTGAACGCTCCGGCGCGAGCGACGGCCATTCTCTAGCGACGCCTCCACGTCCGG  123
            || ||||||| |||| ||||| ||||||| | |||||| |||||| ||||||  ||||||
Sbjct  76   GCTGCCGTGAGCGCTTCGGCGTGAGCGACAGTCATTCTATAGCGATGCCTCCGTGTCCGG  135
Query  124  GAAACCGGC-TGGGGCTGACCTAACCCTTCTCTACGCGTACCTCTG-TTCTCCTTCGGCG  181
            |||||||||  |||||||||||||||||||||||| |||||| |  ||||||||||||||
Sbjct  136  GAAACCGGCGCGGGGCTGACCTAACCCTTCTCTACGAGTACCTATCATTCTCCTTCGGCG  195
Query  182  GGCTCGTCTCGCCGCTGGAACTCACGAACCAACTTGCATTTAGCATTATCTGTTCTGATA  241
            ||  |  | |||||||||||||| | |||||||||||||||||||||| ||||||||||
Sbjct  196  GGG-CAACCCGCCCGCTGGAACTTAAGAACCAACTTGCATTTAGCATTACCTGTTCTGATA  254
Query  242  ACAATCAATTGTTACAACTTTCAACAATGGATCTCTTGGCTCTGGCATCGATGAAGAACG  301
            ||||| |||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  255  ACAATTAATTATTACAACTTTCAACAATGGATCTCTTGGCTCTGGCATCGATGAAGAACG  314
Query  302  CAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC  361
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  315  CAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC  374
Query  362  GCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTACACCCTC  421
            |||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  375  GCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATCTACACCCTC  434
Query  422  AAGCACTGCTTGGTGTTGGGCGTCTGTCCCGCCG-CGCGCGTGGACTCGCCCCAAAGGCA  480
            ||||||||||||||||||||||||||||||||  |||||||||||||||||||||| ||
Sbjct  435  AAGCACTGCTTGGTGTTGGGCGTCTGTCCCGCCTTCGCGCGTGGACTCGCCCCAAAGTCA  494
Query  481  TTGGCAGCGGTCTCTGGCACCTCAACGCGCAGTACATTGCGTACGTTGGG-TGCTGCAGG  539
            |||||||||||||||||||||||||||||||||| ||||| | ||||| ||| |  ||
Sbjct  495  TTGGCAGCGGTCTCTGGCACCTCAACGCGCAGTACAATGCGTTCATTGGGGTGCCGTGGG  554
Query  540  ACGCAACCACAAAGCTAATCACCGTCTTTGACCTCGGATCAGGTAGGGATACCCGCTGAA  599
             |||  |||  |||||| | |||||||||||||||||||||||||||||||||||||||
Sbjct  555  -CGCGTCCAT-AAGCTATTTACCGTCTTTGACCTCGGATCAGGTAGGGATACCCGCTGAA  612
Query  600  CTTAAGCATATCAATAAGCCGGAGGAA                                  626
            |||||||||||||||||| |||||||
Sbjct  613  CTTAAGCATATCAATAAGC-GGAGGAA                                  638
```

Dothideomycetes sp. genotype 189 isolate FL0014 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
Sequence ID: gi|387352752|gb|JQ759890.1|Length: 1134Number of Matches: 1
Score   Expect  Identities     Gaps    Strand
1101 bits(596) 0.0     596/596(100%)  0/596(0%)      Plus/Plus
Query  52   GATCATTACAAGTTGAAACGGTTGCCCTCGCGGTGACCGGTTCTTCAAACCTCTGCGTAC  111
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GATCATTACAAGTTGAAACGGTTGCCCTCGCGGTGACCGGTTCTTCAAACCTCTGCGTAC  60
Query  112  CAAACCTTTCAGTTGCCTCCGGCGGCCCTGGGCCGACGCGGCGCGCGACCTCCCCGTTCG  171
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CAAACCTTTCAGTTGCCTCCGGCGGCCCTGGGCCGACGCGGCGCGCGACCTCCCCGTTCG  120
Query  172  CgggggggCCGCTCCTCGCGGCGGACCACCCGCCGGGCGGTCATAAACAAAACCTTTTGT  231
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CGGGGGGGCCGCTCCTCGCGGCGGACCACCCGCCGGGCGGTCATAAACAAAACCTTTTGT  180
Query  232  CGAGATGGCATCGTCTAATTTCTTCATAACACAATATGAAATACAACTTTTAACAATGGA  291
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  CGAGATGGCATCGTCTAATTTCTTCATAACACAATATGAAATACAACTTTTAACAATGGA  240
Query  292  TCTCTTGGCTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCA  351
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  TCTCTTGGCTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAACTAGTGTGAATTGCA  300
Query  352  GATTTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCTCTTGGTATTCCTCGAGGC  411
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  GATTTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCTCTTGGTATTCCTCGAGGC  360
Query  412  ATGCCTGTTCGAGCGTCGTTACGCCCCTCAAGCGCAAGCTTGGTGTTGGGGATCGCCCCT  471
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  ATGCCTGTTCGAGCGTCGTTACGCCCCTCAAGCGCAAGCTTGGTGTTGGGGATCGCCCCT  420
Query  472  GAGATACGGCGGCGGCCCTTAAATGCATCGGCGGTGCTGGTGTCAGCCCGGAGCGCAGCA  531
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GAGATACGGCGGCGGCCCTTAAATGCATCGGCGGTGCTGGTGTCAGCCCGGAGCGCAGCA  480
Query  532  GACATGCGGCTTCCAGGCGACCACGCGCCCGCCGGACAACGACCCGACTTTCAAACGTCG  591
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GACATGCGGCTTCCAGGCGACCACGCGCCCGCCGGACAACGACCCGACTTTCAAACGTCG  540
Query  592  ACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA      647
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  ACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA      596
```

Fungal endophyte sp. g88 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
Sequence ID: gi|300872433|gb|HM537063.1|Length: 573Number of Matches: 1
Score   Expect  Identities     Gaps    Strand
994 bits(538)  0.0     552/559(99%)   0/559(0%)      Plus/Plus
Query  2    GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACATTCA  61
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
```

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Sbjct  15   GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACATTCA  74
Query  62   GTAGCCTAGCTACTTGTTTACACCCTTGTTTTTTGGCGTACCTATCGTTTCCTCGGCAGG  121
            |||||| |||||||||||||||||||||||| |||||||||||||||||||||| ||
Sbjct  75   GTAGCCCAGCTACTTGTTTACACCCTTGTTTTTTTGCGTACCTATCGTTTCCTCGGCGGG  134
Query  122  CTTGCCTGCCGGCTGGACACCTTTATAACCTTTTTAAATCTTCAATCAGCGTCTGAATAA  181
            |||||||||||| |||||| ||||||||||||||||||||||||||||||||||||| ||
Sbjct  135  CTTGCCTGCCGGTTGGACAACTTTATAACCTTTTTAAATCTTCAATCAGCGTCTGAACAA  194
Query  182  TATACAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC  241
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  195  TATACAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC  254
Query  242  AGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  255  AGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG  314
Query  302  CACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCA  361
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  315  CACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCA  374
Query  362  AGCTTTGCTTGGTGTTGGGTGCTTGTCTTTTTGTGAAGACTCACCTCAAAGTCATTGGCA  421
            |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct  375  AGCTTTGCTTGGTGTTGGGTGCTTGTCTTTTTGTTAAGACTCACCTCAAAGTCATTGGCA  434
Query  422  GCCAGTGTTTTGGTAGTAAGCGCAGCACATTTTGCGTCTTGGTCCCTCAACAGCGGCATC  481
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  435  GCCAGTGTTTTGGTAGTAAGCGCAGCACATTTTGCGTCTTGGTCCCTCAACAGCGGCATC  494
Query  482  CATCAAGCCATTTTCTCACTTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAG  541
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  495  CATCAAGCCATTTTCTCACTTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAG  554
Query  542  CATATCAATAAGCGGAGGA                                          560
            ||||||||||||||||||
Sbjct  555  CATATCAATAAGCGGAGGA                                          573
```

Fungal sp. ARIZ B233 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: gi|223006334|gb|FJ612966.1|Length: 563Number of Matches: 1

Score Expect Identities Gaps Strand
1037 bits(561) 0.0 561/561(100%) 0/561(0%) Plus/Plus

```
Query  1    GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTAAAGAGT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3    GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTAAAGAGT  62
Query  61   TATAACAACTCCCAAACCCCTGTGAACATACCTCATGTTGCCTCGGCAGGTCGCGCCTCG  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  63   TATAACAACTCCCAAACCCCTGTGAACATACCTCATGTTGCCTCGGCAGGTCGCGCCTCG  122
Query  121  GTGCCCTGCCGGCGGCCCACGAAACTCTGTTTAGCATTAAATTCTGAACTTATAACTAAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123  GTGCCCTGCCGGCGGCCCACGAAACTCTGTTTAGCATTAAATTCTGAACTTATAACTAAA  182
Query  181  TCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183  TCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAA  242
Query  241  ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  243  ATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATT  302
Query  301  GCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCCT  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  303  GCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCCT  362
Query  361  CGTTGCTTAGCGTTGGGAGCCTACAAGCACTGTAGCTCCCCAAAGTTAGTGGCGGAGTCG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  363  CGTTGCTTAGCGTTGGGAGCCTACAAGCACTGTAGCTCCCCAAAGTTAGTGGCGGAGTCG  422
Query  421  GTTCACACCCCAGACGTAGTAAGATTTCACCTCGCCTGTAGTTGGACCGGTCCCCTGCCG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  423  GTTCACACCCCAGACGTAGTAAGATTTCACCTCGCCTGTAGTTGGACCGGTCCCCTGCCG  482
Query  481  TAAAACACATAATTTTCTCAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  483  TAAAACACATAATTTTCTCAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTA  542
Query  541  AGCATATCAATAAGCGGAGGA                                        561
            ||||||||||||||||||||
Sbjct  543  AGCATATCAATAAGCGGAGGA                                        563
```

Fusarium equiseti isolate H02-765S 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: gi|192758036|gb|EU595566.1|Length: 1156Number of Matches: 1

Score Expect Identities Gaps Strand
1059 bits(573) 0.0 573/573(100%) 0/573(0%) Plus/Plus

```
Query  4    GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGT  63
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGT  60
Query  64   TTACAACTCCCAAACCCCTGTGAACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGC  123
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TTACAACTCCCAAACCCCTGTGAACATACCTATACGTTGCCTCGGCGGATCAGCCCGCGC  120
Query  124  CCCGTAAAACGGGACGGCCCGCCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTG  183
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  121  CCCGTAAAACGGGACGGCCCGCCCGAGGACCCCTAAACTCTGTTTTTAGTGGAACTTCTG  180
Query  184  AGTAAAACAAACAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT  243
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  AGTAAAACAAACAAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT  240
Query  244  GAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT  303
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  GAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT  300
Query  304  CTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTT  363
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  CTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTT  360
Query  364  CAACCCTCAAGCTCAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATT  423
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CAACCCTCAAGCTCAGCTTGGTGTTGGGACTCGCGGTAACCCGCGTTCCCCAAATCGATT  420
Query  424  GGCGGTCACGTCGAGCTTCCATAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCG  483
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GGCGGTCACGTCGAGCTTCCATAGCGTAGTAATCATACACCTCGTTACTGGTAATCGTCG  480
Query  484  CGGCCACGCCGTAAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCG  543
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  CGGCCACGCCGTAAAACCCCAACTTCTGAATGTTGACCTCGGATCAGGTAGGAATACCCG  540
Query  544  CTGAACTTAAGCATATCAATAAGCGGAGGAAAA                            576
            ||||||||||||||||||||||||||||||||
Sbjct  541  CTGAACTTAAGCATATCAATAAGCGGAGGAAAA                            573
```

Guignardia mangiferae isolate MFUCC120015 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|528747898|gb|KC686598.1|Length: 882Number of Matches: 1

```
Score   Expect  Identities     Gaps    Strand
1190 bits(644) 0.0     659/666(99%)   1/666(0%)      Plus/Plus
Query  5    GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGTGA  64
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||  |
Sbjct  122  GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGAAA  181
Query  65   TGTAATAACTTCTATTGTAAGGTTCCAGAGTAGGCGCTACAACGCCGAAATGACCTTCTC  124
            |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  182  TGTAATAACTTCTATTGAAAGGTTCCAGAGTAGGCGCTACAACGCCGAAATGACCTTCTC  241
Query  125  ACCCTTGTGTACTCACTATGTTGTTTTGGCGGGTCGACCTGGTTCCGACCCATGCGGCCG  184
            |||| |||||||||||||||| |||||||||||||||||||||||||||| |||||||
Sbjct  242  ACCCCTGTGTACTCACTATGTTGCTTTGGCGGGTCGACCTGGTTCCGACCCAGGCGGCCG  301
Query  185  GCGCCCCCAGCCTTAACTGGCCAGGACGCCCGGCTAAGTGCCCGCCAGTATACAAAACTC  244
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  302  GCGCCCCCAGCCTTAACTGGCCAGGACGCCCGGCTAAGTGCCCGCCAGTATACAAAACTC  361
Query  245  AAGAATTCATATTGTGAAGTCCTGATATATCATTTAATTGATTTAAAACTTTCAACAACG  304
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  362  AAGAATTCATATTGTGAAGTCCTGATATATCATTTAATTGATTTAAAACTTTCAACAACG  421
Query  305  GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG  364
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  422  GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTG  481
Query  365  CAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGG  424
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  482  CAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGG  541
Query  425  GCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCAACGTCCG  484
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  542  GCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCAACGTCCG  601
Query  485  CTGCCGGACGTGCCTTGAAGACCTCGGCGACGGCGTCCTAGCCTCGAGCGTAGTAGTAAA  544
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  602  CTGCCGGACGTGCCTTGAAGACCTCGGCGACGGCGTCCTAGCCTCGAGCGTAGTAGTAAA  661
Query  545  ATATCTCGCTTTGGAGTGCTGGGCGACGGCCGCCGGACAATCGACCTTCGGTCTATTTTT  604
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  662  ATATCTCGCTTTGGAGTGCTGGGCGACGGCCGCCGGACAATCGACCTTCGGTCTATTTTT  721
Query  605  CCAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAG-CG  663
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  722  CCAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGGCG  781
Query  664  GAGGAA                                                        669
            ||||||
Sbjct  782  GAGGAA                                                        787
```

Guignardia mangiferae isolate XSD-43 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gb|EU273524.1|Length: 663Number of Matches: 1

```
Score   Expect  Identities     Gaps    Strand
1192 bits(645) 0.0     647/648(99%)   0/648(0%)      Plus/Plus
Query  117  GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGAAA  176
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACTGAAA  60
Query  177  TGTAATAACTTCTATTGAAAGGTTCCAGAGTAGGCGCTACAACGCCGAAATGACCTTCTC  236
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TGTAATAACTTCTATTGAAAGGTTCCAGAGTAGGCGCTACAACGCCGAAATGACCTTCTC  120
Query  237  ACCCTTGTGTACTCACTATGTTGCTTTGGCGGGTCGACCTGGTTCCGACCCAGGCGGCCG  296
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct  121  ACCCTTGTGTACTCACTATGTTGCTTTGGCGGGTCGACCTGGTTCCGACCCAGGCGGCCG  180
Query  297  GCGCCCCCAGCCTTAACTGGCCAGGACGCCCGGCTAAGTGCCCGCCAGTATACAAAACTC  356
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GCGCCCCCAGCCTTAACTGGCCAGGACGCCCGGCTAAGTGCCCGCCAGTATACAAAACTC  240
Query  357  AAGAATTCATTTTGTGAAGTCCTGATATATCATTTAATTGATTAAAACTTTCAACAACGG  416
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AAGAATTCATTTTGTGAAGTCCTGATATATCATTTAATTGATTAAAACTTTCAACAACGG  300
Query  417  ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC  476
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC  360
Query  477  AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGG  536
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGG  420
Query  537  CATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCAACGTCCGC  596
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  CATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTATTGGGCAACGTCCGC  480
Query  597  TGCCGGACGTGCCTTGAAGACCTCGGCGACGGCGTCCTAGCCTCGAGCGTAGTAGTAAAA  656
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  TGCCGGACGTGCCTTGAAGACCTCGGCGACGGCGTCCTAGCCTCGAGCGTAGTAGTAAAA  540
Query  657  TATCTCGCTTTGGAGTGCTGGGCGACGGCTGCCGGACAATCGACCTTCGGTCTATTTTTC  716
            |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  541  TATCTCGCTTTGGAGTGCTGGGCGACGGCCGCCGGACAATCGACCTTCGGTCTATTTTTC  600
Query  717  CAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATA              764
            |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  CAAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATA              648
```

Letendraea helminthicola strain F158 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|406780888|gb|JQ026217.1|Length: 724Number of Matches: 1
Score Expect Identities Gaps Strand
1221 bits(661) 0.0 667/670(99%) 0/670(0%) Plus/Plus

```
Query  14   CGTAACAAGGTTTTCGTAGGGGAACCTGCGGAAGGATCATTACATTTTCACAACAACGAT  73
            |||||||||||||| |||||| ||||||||||||||||||||||||||||||||||||
Sbjct  1    CGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACATTTTCACAACAACGAT  60
Query  74   GGCGTCGTCCTTAGAACCGTCTCCGTGCGGCTCGGGGCGGCGTTTCATCAGCGGGCACGT  133
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GGCGTCGTCCTTAGAACCGTCTCCGTGCGGCTCGGGGCGGCGTTTCATCAGCGGGCACGT  120
Query  134  CGCGGCTTCCTGTTTTCAGGAAGTAATCCCATCTAGAATGGACTCACGCGGGGTCGTCTG  193
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  CGCGGCTTCCTGTTTTCAGGAAGTAATCCCATCTAGAATGGACTCACGCGGGGTCGTCTG  180
Query  194  AATCCTTAACTTTACGAGAACTCCCCATACTCCTTCGGTGGGGTGACCTGCCGTTGGAAC  253
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  AATCCTTAACTTTACGAGAACTCCCCATACTCCTTCGGTGGGGTGACCTGCCGTTGGAAC  240
Query  254  CAACAAAAACCttttttttGCATCTAGCATTACCTGTTCTGATACAAACAATCGTTACAAC  313
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CAACAAAAACCTTTTTTTGCATCTAGCATTACCTGTTCTGATACAAACAATCGTTACAAC  300
Query  314  TTTCAACAATGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGT  373
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  TTTCAACAATGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGT  360
Query  374  AGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGG  433
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  AGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTTGG  420
Query  434  TATTCCATGGGGCATGCCTGTTCGAGCGTCATCTACACCCTCAAGCTCTGCTTGGTGTTG  493
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  TATTCCATGGGGCATGCCTGTTCGAGCGTCATCTACACCCTCAAGCTCTGCTTGGTGTTG  480
Query  494  GGCGTCTGTCCCGCCTCCGCGCGTGGACTCGCCCCAAATTCATTGGCAGCGGTCTTCTTG  553
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GGCGTCTGTCCCGCCTCCGCGCGTGGACTCGCCCCAAATTCATTGGCAGCGGTCTTCTTG  540

Query  554  CCCCCTCTCGCGCAGCACATTGCGTTTCTCGAGGGTGGCGGGCCGCGTCCACGAAGCAAC  613
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  CCCCCTCTCGCGCAGCACATTGCGTTTCTCGAGGGTGGCGGGCCGCGTCCACGAAGCAAC  600
Query  614  ATTCACCGTCTTTGACCTCGGATCAGGTAGGGATACCCGCTGAAATTAAGCATATCAATA  673
            |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  601  ATTCACCGTCTTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATA  660
Query  674  AGCGGAGGAA                                                    683
            ||||||||||
Sbjct  661  AGCGGAGGAA                                                    670
```

Minimidochium sp. TMS-2011 voucher MS3p_50-45 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|317383295|gb|HQ630974.1|Length: 581Number of Matches: 1
Score Expect Identities Gaps Strand
909 bits(492) 0.0 551/577(95%) 13/577(2%) Plus/Plus

```
Query  5    GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACAGGAC  64
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16   GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACAGGAC  75
Query  65   GTCACACTCCCGTAATACCACTGTGAATCTTACCTACCGTCGTTGCCTCGGCGGGGTCGG  124
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            |||| ||||||||||||||||||||||||||||||||||||||||||||||||||  | |
Sbjct  76   GTCATACTCCCGTAATACCACTGTGAATCTTACCTACCGTCGTTGCCTCGGCGGG--C-G  132
Query  125  TCCCCCTCGGGGGGCGCCCGCCGGCGGCCCGCATACTCTGTCTCAGCGTGTTGGCATCTC  184
                 |||   | ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  133  -----CTC--CGCGCGCCCGCCGGCGGCCCGCATACTCTGTCTCAGCGTGTTGGCATCTC  185
Query  185  CGAGTACAATACAAACGAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT  244
            ||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  186  CGAGTATTATACAAACGAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGAT  245
Query  245  GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT  304
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  246  GAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAAT  305
Query  305  CTTTGAACGCACATTGCGCCCGCCAGTATCCTGGCGGGCATGCCTGTTCGAGCGTCATTT  364
            ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  306  CTTTGAACGCACATTGCGCCCGCCAGCATCCTGGCGGGCATGCCTGTTCGAGCGTCATTT  365
Query  365  CAACCCTCAAGCCCCCGCGGCTTGGTGTTGGAGGCCTGCGCACCGCAGCCTCCCAAAGAC  424
            |||||||||||||||||||||||||||| | ||||||||||||||| || ||||||
Sbjct  366  CAACCCTCAAGCCCCCGCGGCTTGGTGTTGGGGACCTGCGCACCGCAGCCCCCTAAAGAC  425
Query  425  AGCGGCGGGCGTGGCCTGGCACCGAACGTAGTAGACTCTCTCTCGTTCGCGGTCCAGGGC  484
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||  |
Sbjct  426  AGCGGCGGGCGTGGCCTGGCACCGAACGTAGTAGACTCTCTCTCGTTCGCGGTCCAGTTC  485
Query  485  GCGCTTCCGGCCGGTAAACCCCCATCTTTTA-CG-TGGTTGACCTCGGATCAGGTAGGAA  542
            |||| |||||||||||||||||||||||| |  ||||||||||||||||||||||||||
Sbjct  486  GCGC-TCCGGCCGGTAAACCCCCATCTTTTAACAATGGTTGACCTCGGATCAGGTAGGAA  544
Query  543  TACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA                         579
            |||||||||||||||||||||||||||||||||||||
Sbjct  545  TACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA                         581
```

Montagnulaceae sp. HJ15H 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|485185712|gb|KC291642.1|Length: 721Number of Matches: 1

```
Score   Expect  Identities     Gaps    Strand
1325 bits(717) 0.0    720/721(99%)   1/721(0%)      Plus/Plus
Query  40   TGCGGAGGGATCATTATTCATTACAGTAAGCCTATGGCTTCGGGGGCGAACAAGCCAGGC  99
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2    TGCGGAGGGATCATTATTCATTACAGTAAGCCTATGGCTTCGGGGGCGAACAAGCCAGGC  61
Query  100  GCGGTCGACCCTTCGGGGCGTGGCAGCGTCGCGGCTCCCCGAATCGTTATGCTTTAGCGG  159
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  62   GCGGTCGACCCTTCGGGGCGTGGCAGCGTCGCGGCTCCCCGAATCGTTATGCTTTAGCGG  121
Query  160  GTGTGTTGCGGCCCTCGAGGCCCCGGACCGGCTCGACAGTGGTCAGCCGGCACGGGAACC  219
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  122  GTGTGTTGCGGCCCTCGAGGCCCCGGACCGGCTCGACAGTGGTCAGCCGGCACGGGAACC  181
Query  220  AGCTCCGGGAGGTGTAGGGCTCCTCGTGGAGCCCTTTCACGCGCACATGACTGAATCCTT  279
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  182  AGCTCCGGGAGGTGTAGGGCTCCTCGTGGAGCCCTTTCACGCGCACATGACTGAATCCTT  241
Query  280  ACTCTACTCGTACCTCTCGTTCTCCCTCGGCGGGGCGACCTGCCGTGGGAACCGCAAAAC  339
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  242  ACTCTACTCGTACCTCTCGTTCTCCCTCGGCGGGGCGACCTGCCGTGGGAACCGCAAAAC  301
Query  340  GAACCCTTCTTTGCATCTAGCATTACCTGTTCCGATACCCACAATCGTTACAACTTTCAA  399
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  302  GAACCCTTCTTTGCATCTAGCATTACCTGTTCCGATACCCACAATCGTTACAACTTTCAA  361
Query  400  CAATGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTG  459
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  362  CAATGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTG  421
Query  460  AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTCGGTATTCC  519
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  422  AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCTCGGTATTCC  481
Query  520  GTGGGGCATGCCTGTTCGAGCGTCATCTACACCCTCAAGCTCTGCTTGGTGTTGGGCGTC  579
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  482  GTGGGGCATGCCTGTTCGAGCGTCATCTACACCCTCAAGCTCTGCTTGGTGTTGGGCGTC  541
Query  580  TGTCCCGCCTCCTTCGCGCGCGGACTCGCCCCAAATCCATTGGCGGCGGCCCTTGCCTCC  639
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  542  TGTCCCGCCTCCTTCGCGCGCGGACTCGCCCCAAATCCATTGGCGGCGGCCCTTGCCTCC  601
Query  640  CTCTCGCGCAGCACATTGCGCTTCTCGAGGCGCGGCGGACCGCGTCCAGCAAGCAACCGT  699
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  602  CTCTCGCGCAGCACATTGCGCTTCTCGAGGCGCGGCGGACCGCGTCCAGCAAGCAACCGT  661
Query  700  CACACCGTCTTTGACCTCGGATGCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATA  759
            |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  662  CACACCGTCTTTGACCTCGGAT-CAGGTAGGGATACCCGCTGAACTTAAGCATATCAATA  720
Query  760  A                                                             760
            |
Sbjct  721  A                                                             721
```

Myrothecium sp. 2 TMS-2011 voucher SC8d10p9-5 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|317383388|gb|HQ631067.1|Length: 622Number of Matches: 1

```
Score   Expect  Identities     Gaps    Strand
1094 bits(592) 0.0    602/607(99%)   0/607(0%)      Plus/Plus
Query  4    GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGT  63
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16   GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGT  75
Query  64   TTACAAACTCCCAACCCTATGTGAACCTTACCTATCGTTGCTTCGGCGGGCTTAGCCCCC  123
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  76   TTACAAACTCCCAACCCTATGTGAACCTTACCTATCGTTGCTTCGGCGGGCTTAGCCCCC  135
Query  124  GCGCCCTCGCCGGCGCCGGGAAACAGGCGCCCGCCGGAGACCCAAACTCAATGTTTTTCA  183
            ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  136  GCGCCTTCGCCGGCGCCGGGAAACAGGCGCCCGCCGGAGACCCAAACTCAATGTTTTTCA  195
Query  184  TGCAGTATTATCTGAGTGGCAAACGCAAAAATAAATCAAAACTTTTAACAACGGATCTCT  243
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  196  TGCAGTATTATCTGAGTGGCAAACGCAAAAATAAATCAAAACTTTTAACAACGGATCTCT  255
Query  244  TGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATT  303
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  256  TGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATT  315
Query  304  CAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCGAGTATTCTCGCGGGCATGCC  363
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  316  CAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCGAGTATTCTCGCGGGCATGCC  375
Query  364  TGTTCGAGCGTCATTTCAACCCTCAGAGCCCGTCTGCTTGTTCAGGCGCGGCGCTGGTGT  423
            ||| |||||||||||||||||||||||| || ||||||||||||||||||||||||||||||
Sbjct  376  TGTCCGAGCGTCATTTCAACCCTCAGAGCTCGCCTGCTTGTTCAGGCGCGGCGCTGGTGT  435
Query  424  TGGGGATCGGCCTAAACGCCGTCCCCCAAATACAGTGGCGGTCTCGCTGCAGCCTCCCCT  483
            ||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  436  TGGGGATCGGCCTAACCGCCGTCCCCCAAATACAGTGGCGGTCTCGCTGCAGCCTCCCCT  495
Query  484  GCGTAGTAGCAACACTCGCATGCGGAGCGCGGCGCGGCCACGCCGTAAAACCCCCGACTT  543
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  496  GCGTAGTAGCAACACTCGCATGCGGAGCGCGGCGCGGCCACGCCGTAAAACCCCCGACTT  555
Query  544  TCTGAACGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGC  603
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  556  TCTGAACGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGC  615
Query  604  GGAGGAA                                                       610
            |||||||
Sbjct  616  GGAGGAA                                                       622
```

Nemania primolutea isolate 91102001 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|122935533|gb|EF026121.1|Length: 582Number of Matches: 1

```
Score   Expect  Identities     Gaps    Strand
1064 bits(576) 0.0    580/582(99%)   0/582(0%)      Plus/Plus
Query  28   TCCGTTGGTGAACCAGCGGAGGGATCATTAAAGAGTTTTCTACAACTCCCAAACCCCTGT  87
            ||||| |||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TCCGTAGGTGAACCTGCGGAGGGATCATTAAAGAGTTTTCTACAACTCCCAAACCCCTGT  60
Query  88   GAACATACCTTCTGTTGCCTCGGCAGGCCTCGCCTACCCTCGTAGCCCCCTACACCGTAG  147
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GAACATACCTTCTGTTGCCTCGGCAGGCCTCGCCTACCCTCGTAGCCCCCTACACCGTAG  120
Query  148  GGCCTACGCCGGGTGGTGCGCGGACCCTGCCGGCGGCCCGCGAAACTCTGTTTAGCACTG  207
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GGCCTACGCCGGGTGGTGCGCGGACCCTGCCGGCGGCCCGCGAAACTCTGTTTAGCACTG  180
Query  208  AATCTCTGAACATATAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGG  267
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  AATCTCTGAACATATAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTGG  240
Query  268  CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC  327
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATC  300
Query  328  ATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGC  387
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  ATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAGC  360
Query  388  GTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGGGAGCCTACGGCAGCGTAGCTCC  447
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGGGAGCCTACGGCAGCGTAGCTCC  420
Query  448  CCAAAGTTAGTGGCGTGGTCGGTTCACACTCCAGACGTAGTAGATTTTCGTCTCGCCTGT  507
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  CCAAAGTTAGTGGCGTGGTCGGTTCACACTCCAGACGTAGTAGATTTTCGTCTCGCCTGT  480
Query  508  AGTTGGACCGGTCCCCTGCCGTAAAACACCCCAATTCTAAAAGGTTGACCTCGGATCAGG  567
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  AGTTGGACCGGTCCCCTGCCGTAAAACACCCCAATTCTAAAAGGTTGACCTCGGATCAGG  540
Query  568  TAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA                    609
            |||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  TAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA                    582
```

Nemania primolutea isolate FG9 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gb|KC845930.1|Length: 511Number of Matches: 1

```
Score   Expect  Identities     Gaps    Strand
944 bits(511)  0.0    511/511(100%)  0/511(0%)      Plus/Plus
Query  1    TGAACATACCTTCTGTTGCCTCGGCAGGCCTCGCCTACCCTCGTAGCCCCCTACACCGTA  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TGAACATACCTTCTGTTGCCTCGGCAGGCCTCGCCTACCCTCGTAGCCCCCTACACCGTA  60
Query  61   GGGCCTACGCCGGGTGGTGCGCGGACCCTGCCGGCGGCCCGCGAAACTCTGTTTAGCACT  120
```

```
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   GGGCCTACGCCGGGTGGTGCGCGGACCCTGCCGGCGGCCCGCGAAACTCTGTTTAGCACT  120
Query  121  GAATCTCTGAACATATAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GAATCTCTGAACATATAACTAAATAAGTTAAAACTTTCAACAACGGATCTCTTGGTTCTG  180
Query  181  GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT  240
Query  241  CATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAG  300
Query  301  CGTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGGGAGCCTACGGCAGCGTAGCTC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  CGTCATTTCAACCCTTAAGCCCCTGTTGCTTAGCGTTGGGAGCCTACGGCAGCGTAGCTC  360
Query  361  CCCAAAGTTAGTGGCGTGGTCGGTTCACACTCCAGACGTAGTAGATTTTCGTCTCGCCTG  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CCCAAAGTTAGTGGCGTGGTCGGTTCACACTCCAGACGTAGTAGATTTTCGTCTCGCCTG  420
Query  421  TAGTTGGACCGGTCCCCTGCCGTAAAACACCCCAATTCTAAAAGGTTGACCTCGGATCAG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  TAGTTGGACCGGTCCCCTGCCGTAAAACACCCCAATTCTAAAAGGTTGACCTCGGATCAG  480
Query  481  GTAGGAATACCCGCTGAACTTAAGCATATCA                               511
            ||||||||||||||||||||||||||||||||
Sbjct  481  GTAGGAATACCCGCTGAACTTAAGCATATCA                               511
```

Paecilomyces sp. TMS-2011 voucher BGd1p19-17 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|317383362|gb|HQ631041.1|Length: 606Number of Matches: 1

```
Score   Expect  Identities     Gaps    Strand
902 bits(488)  0.0    566/601(94%)   15/601(2%)     Plus/Plus
Query  2    GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGTTTGGAACCAGCGGAGGGATCATTACAG  61
            ||||||||||||||||||||||||||||||||   |||||||||||||||||||||||||
Sbjct  16   GGAAGTAAAAGTCGTAACAAGGTCTCCGTTGGT---GAACCAGCGGAGGGATCATTACAG  72
Query  62   AGTTGAAAAACTCCCCCAACCATTGTGAACC-TACCTTCCACCGTTGCTTCGGCGGGCGG  120
            |||||  |||||||||| |||||||||||||| |||||||| ||||||||||||||||||||
Sbjct  73   AGTTGCAAAACTCCCCAAACCATTGTGAACCTTACCTTCAACCGTTGCTTCGGCGGGCGG  132
Query  121  GCCCCAGCGccccccccggcccccccccGGGGGGCGCCCGCCGGAGGATGCACAAACTCTT  180
            |||  |||||  ||||||||||||||| | |||||||||||||||||| | |||||||||
Sbjct  133  GCCACAGCG-CCCCCCCGGCCCCCCCAGCGGGGCGCCCGCCGGAGGATACCCAAACTCTT  191
Query  181  TGT-CATTAGCGGCCTCTCTGAGTCTT---TACTGAATAAGTCAAAACTTTCAACAACGG  236
              | | |||  ||||||||||||||||   ||||||||||||||||||||||||||||||
Sbjct  192  GATACTTTAT-GGCCTCTCTGAGTCTTCTGTACTGAATAAGTCAAAACTTTCAACAACGG  250
Query  237  ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC  296
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  251  ATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC  310
Query  297  AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTACTCTGGCGGG  356
            |||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
Sbjct  311  AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGG  370
Query  357  CATGCCTGTTCGAGCGTCATTTCAACCATCAAGCCCCCGGGCTTGTGTTGGGGACCTGCG  416
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  371  CATGCCTGTTCGAGCGTCATTTCAACCATCAAGCCCCCGGGCTTGTGTTGGGGACCTGCG  430
Query  417  GCTGCCCGCAGGCCCTGAAAAGCAGTGGCGGGCTCGCCGTCACGCCGAGCGTAGTAGCAA  476
            |||||||||||||||||||| |||||||||||||| ||||| |||||||||||||| |
Sbjct  431  GCTGCCCGCAGGCCCTGAAAACCAGTGGCGGGCTCGCTGTCACACCGAGCGTAGTAGC-A  489
Query  477  TCATCTCGCTCAGGGCGTGCTGCGGGCTCCGGCCGTTAAACAACAGCCTCTCAGGCCCAA  536
            |||||||||||||||||||||||||| |||||||||||    ||||||| |  |||||
Sbjct  490  TCATCTCGCTCAGGGCGTGCTGCGGGTTCCGGCCGTTAAA----AGCCTCTAATACCCAA  545
Query  537  GGTTGACCTCGGATCAGGTAGGAAGACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA  596
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  546  GGTTGACCTCGGATCAGGTAGGAAGACCCGCTGAACTTAAGCATATCAATAAGCGGAGGA  605
Query  597  A                                                             597
            |
Sbjct  606  A                                                             606
```

Penicillium sp. HF12230 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|392514877|gb|JQ889696.1|Length: 580Number of Matches: 1

```
Score   Expect  Identities     Gaps    Strand
1018 bits(551) 0.0    567/574(99%)   4/574(0%)      Plus/Plus
Query  1    GAAGT-AAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGT  59
            |||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3    GAAGTAAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGT  62
Query  60   GCGGGCCCTCGCGGCCCAACCTCCCACCCGTGTCTCTCTCTACCTGTTGCTTTGGCGGGC  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  63   GCGGGCCCTCGCGGCCCAACCTCCCACCCGTGTCTCTCTCTACCTGTTGCTTTGGCGGGC  122
Query  120  CCACCGGGGCCACCCGGTCGCCGGGGGACGT-CGTCCCCGGGCCCGCGCCCGCCGAAGCG  178
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
Sbjct  123  CCACCGGGGCCACCCGGTCGCCGGGGGACGTCCGTCCCCGGGCCCGCGCCCGCCGAAGCG  182
Query  179  CTCTGTGAACCCTGATGAAGATGGGCTGTCTGAGTCGAATGAAAATTGTCAAAACTTTCA  238
```

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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183  CTCTGTGAACCCTGATGAAGATGGGCTGTCTGAGTCGAATGAAAATTGTCAAAACTTTCA  242
Query  239  ACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT  298
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  243  ACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGT  302
Query  299  GAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGCATTC  358
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  303  GAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCTGGCATTC  362
Query  359  CGGGGGGCATGCCTGTCCGAGCGTCATTTCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCG  418
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  363  CGGGGGGCATGCCTGTCCGAGCGTCATTTCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCG  422
Query  419  TGGTCCCCCCGGGGACCTGCCCGAAAGGCAGCGGCGACGTCCGTCTGGTCCTCGAGCGTA  478
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  423  TGGTCCCCCCGGGGACCTGCCCGAAAGGCAGCGGCGACGTCCGTCTGGTCCTCGAGCGTA  482
Query  479  TGGGGCTCTGTCACTCGCT-CGGGACGGATCGGCGGAGGTTGGTCACCACCACAGTTTTA  537
            ||||||| |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  483  TGGGGCTTTGTCACTCGCTACGGGACGGATCGGCGGAGGTTGGTCACCACCACAGTTTTA  542
Query  538  CCACGGTTGACCTCGGATCAGGTAGGAGTTACCC                             571
            |||||| |||||||||||||||||| || |||
Sbjct  543  CCACGG-TGACCTCGGATCAGGTAGGATTTCCCC                             575
```

Phoma sp. TMS-2011 voucher MS5p50-9 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|317383284|gb|HQ630963.1|Length: 574Number of Matches: 1

```
Score   Expect  Identities     Gaps    Strand
1026 bits(555) 0.0     557/558(99%)   0/558(0%)      Plus/Plus
Query  1    GAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  17   GAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAG  76
Query  61   TTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTACGTTTCCTCGGT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  77   TTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTACGTTTCCTCGGT  136
Query  121  GGGTTCGCCCACCGATTGGACAAATTTAAACCCTTTGCAGTTGAAATCAGCGTCTGAAAA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  137  GGGTTCGCCCACCGATTGGACAAATTTAAACCCTTTGCAGTTGAAATCAGCGTCTGAAAA  196
Query  181  AACTCAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC  240
            |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  197  AACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC  256
Query  241  AGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  257  AGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACG  316
Query  301  CACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  317  CACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTCA  376
Query  361  AGCTCTGCTTGGTGTTGGGTGTTTGTCTCCTGTAGACTCGCCTTAAAACAATTGGCAGCC  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  377  AGCTCTGCTTGGTGTTGGGTGTTTGTCTCCTGTAGACTCGCCTTAAAACAATTGGCAGCC  436
Query  421  GGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACATCCA  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  437  GGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACATCCA  496
Query  481  AAAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  497  AAAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCA  556
Query  541  TATCAATAAGCGGAGGAA                                            558
            ||||||||||||||||||
Sbjct  557  TATCAATAAGCGGAGGAA                                            574
```

Phoma sp. TMS-2011 voucher MS5p50-9 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|317383284|gb|HQ630963.1|Length: 574Number of Matches: 1

```
Score   Expect  Identities     Gaps    Strand
1033 bits(559) 0.0     559/559(100%)  0/559(0%)      Plus/Plus
Query  2    GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGA  61
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16   GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGA  75
Query  62   GTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTACGTTTCCTCGG  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  76   GTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTACGTTTCCTCGG  135
Query  122  TGGGTTCGCCCACCGATTGGACAAATTTAAACCCTTTGCAGTTGAAATCAGCGTCTGAAA  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  136  TGGGTTCGCCCACCGATTGGACAAATTTAAACCCTTTGCAGTTGAAATCAGCGTCTGAAA  195
Query  182  AAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACG  241
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  196  AAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACG  255
Query  242  CAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  256  CAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC  315
Query  302  GCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTC  361
```

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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  316  GCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTC  375
Query  362  AAGCTCTGCTTGGTGTTGGGTGTTTGTCTCCTGTAGACTCGCCTTAAAACAATTGGCAGC  421
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  376  AAGCTCTGCTTGGTGTTGGGTGTTTGTCTCCTGTAGACTCGCCTTAAAACAATTGGCAGC  435
Query  422  CGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACATCC  481
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  436  CGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACATCC  495
Query  482  AAAAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGC  541
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  496  AAAAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGC  555
Query  542  ATATCAATAAGCGGAGGAA                                           560
            ||||||||||||||||||
Sbjct  556  ATATCAATAAGCGGAGGAA                                           574
```

Phoma sp. P14E4 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
Sequence ID: gi|346654886|gb|JN207285.1|Length: 562Number of Matches: 1
Score   Expect  Identities     Gaps    Strand
1035 bits(560) 0.0     560/560(100%)  0/560(0%)      Plus/Plus
Query  4    GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGA  63
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3    GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGA  62
Query  64   GTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTTCGTTTCCTCGG  123
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  63   GTTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTTCGTTTCCTCGG  122
Query  124  CGGGTCCGCCCGCCGATTGGACACATTCAAACCCTTTGCAGTTGCAATCAGCGTCTGAAA  183
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  123  CGGGTCCGCCCGCCGATTGGACACATTCAAACCCTTTGCAGTTGCAATCAGCGTCTGAAA  182
Query  184  AAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACG  243
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  183  AAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACG  242
Query  244  CAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC  303
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  243  CAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC  302
Query  304  GCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTC  363
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  303  GCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTC  362
Query  364  AAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCCTCTGCGCGCAGACTCGCCTCAAAACAA  423
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  363  AAGCTCTGCTTGGTGTTGGGTGTTTGTCTCGCCTCTGCGCGCAGACTCGCCTCAAAACAA  422
Query  424  TTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGA  483
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  423  TTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGA  482
Query  484  CGACGATCCAAAAGTACATTTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCT  543
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  483  CGACGATCCAAAAGTACATTTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCT  542
Query  544  GAACTTAAGCATATCAATAA                                          563
            ||||||||||||||||||||
Sbjct  543  GAACTTAAGCATATCAATAA                                          562
```

Phoma sp. CPO 10.003 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
Sequence ID: gi|378750663|gb|JQ388280.1|Length: 557Number of Matches: 1
Score   Expect  Identities     Gaps    Strand
1020 bits(552) 0.0     557/559(99%)   2/559(0%)      Plus/Plus
Query  5    GAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAG  64
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAG  60
Query  65   TTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTACGTTTCCTCGGT  124
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTACGTTTCCTCGGT  120
Query  125  GGGTTCGCCCACCGATTGGACAAATTTAAACCCTTTGCAGTTGAAATCAGCGTCTGaaaa  184
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  121  GGGTTCGCCCACCGATTGGACAAATTTAAACCCTTTGCAGTTGAAATCAGCGTCTG-AAA  179
Query  185  aaaCTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACG  244
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  180  AAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACG  239
Query  245  CAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC  304
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  240  CAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC  299
Query  305  GCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTC  364
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  300  GCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTC  359
Query  365  AAGCTCTGCTTGGTGTTGGGTGTTTGTCTCCTGTAGACTCGCCTTAAAACAATTGGCAGC  424
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  360  AAGCTCTGCTTGGTGTTGGGTGTTTGTCTCCTGTAGACTCGCCTTAAAACAATTGGCAGC  419
Query  425  CGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACATCC  484
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            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  420  CGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACATCC  479
Query  485  AAAAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGC  544
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  480  AAAAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGC  539
Query  545  ATATCAATAAGCCGGAGGA                                           563
            |||||||||||| ||||||
Sbjct  540  ATATCAATAAGC-GGAGGA                                           557
```

Phoma sp. CPO 10.003 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|378750663|gb|JQ388280.1|Length: 557Number of Matches: 1

```
Score   Expect  Identities     Gaps    Strand
1024 bits(554) 0.0    557/558(99%)   1/558(0%)      Plus/Plus
Query  6    GAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAG  65
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    GAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAG  60
Query  66   TTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTACGTTTCCTCGGT  125
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TTGTAGGCTTTGCCTGCTATCTCTTACCCATGTCTTTTGAGTACCTTACGTTTCCTCGGT  120
Query  126  GGGTTCGCCCACCGATTGGACAAATTTAAACCCTTTGCAGTTGAAATCAGCGTCTGaaaa  185
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
Sbjct  121  GGGTTCGCCCACCGATTGGACAAATTTAAACCCTTTGCAGTTGAAATCAGCGTCTG-AAA  179
Query  186  aaaCTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACG  245
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  180  AAACTTAATAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACG  239
Query  246  CAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC  305
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  240  CAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAAC  299
Query  306  GCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTC  365
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  300  GCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACCTTC  359
Query  366  AAGCTCTGCTTGGTGTTGGGTGTTTGTCTCCTGTAGACTCGCCTTAAAACAATTGGCAGC  425
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  360  AAGCTCTGCTTGGTGTTGGGTGTTTGTCTCCTGTAGACTCGCCTTAAAACAATTGGCAGC  419
Query  426  CGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACATCC  485
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  420  CGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCTTTGCACTCATAACGACGACATCC  479
Query  486  AAAAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGC  545
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  480  AAAAGTACATTTTTACACTCTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGC  539
Query  546  ATATCAATAAGCGGAGGA                                           563
            ||||||||||||||||||
Sbjct  540  ATATCAATAAGCGGAGGA                                           557
```

Pleosporales sp. 3 TMS-2011 voucher SC8d10p9-6 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Sequence ID: gi|317383336|gb|HQ631015.1|Length: 588Number of Matches: 1

```
Score   Expect  Identities     Gaps    Strand
1087 bits(565) 0.0    567/568(99%)   0/568(0%)      Plus/Plus
Query  3    GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACATTCA  62
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16   GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACATTCA  75
Query  63   GTAGCCTAGCTACTTGTTTACACCCTTGttttttttGCGCACTTCATTGTATCCCTCGGC  122
            |||||||||||||||||||||||||||||||||||| ||||||||||||||
Sbjct  76   GTAGCCTAGCTACTTGTTTACACCCTTGTTTTTTTTGCGCACTTCACTGTATCCCTCGGC  135
Query  123  GGGCTTGCTCGCCGGTTGGACAACATTTATAACCTTTTTTAATCTTCAATCAGCGTCTGA  182
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  136  GGGCTTGCTCGCCGGTTGGACAACATTTATAACCTTTTTTAATCTTCAATCAGCGTCTGA  195
Query  183  ATTATATTTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGA  242
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  196  ATTATATTTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGA  255
Query  243  ACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTG  302
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  256  ACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTG  315
Query  303  AACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACC  362
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  316  AACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGTACC  375
Query  363  CTCAAGCTTTGCTTGGTGTTGGGCGTCTTGTCTACCTCTTTAGCGGGTAGACTCGCCTTA  422
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  376  CTCAAGCTTTGCTTGGTGTTGGGCGTCTTGTCTACCTCTTTAGCGGGTAGACTCGCCTTA  435
Query  423  AAGTAATTGGCAGCCAGTGTTTTTGGCAGTAAGCGCAGCACATTTTGCGTCTTAGTCCCT  482
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  436  AAGTAATTGGCAGCCAGTGTTTTTGGCAGTAAGCGCAGCACATTTTGCGTCTTAGTCCCT  495
Query  483  AAACAGTGGCATCCACAAAGCCTCTTTCTCACTTTTGACCTCGGATCAGGTAGGGATACC  542
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  496  AAACAGTGGCATCCACAAAGCCTCTTTCTCACTTTTGACCTCGGATCAGGTAGGGATACC  555
Query  543  CGCTGAACTTAAGCATATCAATAAGCGG                                  570
```

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            ||||||||||||||||||||||||||
Sbjct  556  CGCTGAACTTAAGCATATCAATAAGCGG                                  583
```

Xylaria sp. P055 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence
Sequence ID: gi|126131223|gb|EF423534.1|Length: 589Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
883 bits(478)	0.0	561/599(94%)	13/599(2%)	Plus/Plus

```
Query  15   TCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACAGAGTTTATCATAACT  74
            ||||||||||||||||||||||||| |||||||||| ||||| |||| || | ||||||
Sbjct  1    TCGTAACAAGGTCTCCGTTGGTGAAC-AGCGGAGGGA-CATTAAAGAG-TTCT-ATAACT  56
Query  75   CCCAAACCCATGTGAACATACCTTACGTTGCCTCGGCAGGACGTGCTTACCCCGTAGCGC  134
            |||||||||||||||||||| |||||||||||||||| || || ||||||||||||
Sbjct  57   CCCAAACCCATGTGAACATACCATACGTTGCCTCGGCAGGTCGCGCCTACCCCGTAGCGT  116
Query  135  CCTACCCTGTAGGGCCTACCCGGGAAGGC-CGGGTAACCCTGCCAATGGCCCACGAAACT  193
            |||||||| |||| |||||||||  || | ||||||| ||||||   |||||| ||||||
Sbjct  117  CCTACCCTATAGGACCTACCCGG-TAGACGCGGGTAAGCCTGCCGGCGGCCCAAGAAACT  175
Query  194  CTGTTTAGTATTGAATTCTGAACTTATAACTAAATAAAGTTAAAACTTTCAACAACGGAT  253
            ||||||||||||||||||||| |||||||||| |||||||||||||||||||||||||
Sbjct  176  CTGTTTAGTATTGAATTCTGAACCTATAACTAAAT-AAGTTAAAACTTTCAACAACGGAT  234
Query  254  CTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG  313
            |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  235  CTCTTGGTTCTGGCATCGATGAAGAAC-CAGCGAAATGCGATAAGTAATGTGAATTGCAG  293
Query  314  AATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCA  373
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  294  AATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTATTCTAGTGGGCA  353
Query  374  TGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCCTCGTTGCTTAGTGTTGGGAGCCTACG  433
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  354  TGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCCTCGTTGCTTAGTGTTGGGAGCCTACG  413
Query  434  GTGCT-GTAGCTCTTCAAAGTTAGTGGCGGAGTCGGTTCACACTCTAGACGTAGTAAATT  492
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Sbjct  414  GTACCCGTAGCTCCTCAAAGTTAGTGGCGGAGTCGGTTCACACTCTAGACGTAGTAA-TT  472
Query  493  TTCACCTCGCCTGT-AGTTGGACCGGTCCCTTGCCGTAAAACACCTTAATTATCTAAAGG  551
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Sbjct  473  TT-ATCTCGCCTATCAGTTGGACCGGTCCCCTGCCGTAAAACACCCCAATT-TCTAAAGG  530
Query  552  TTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA   610
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Sbjct  531  TTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA   589
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APPENDIX 3

Zone of inhibition values demonstrated by marine derived fungi against test bacteria in the plug assay

Table 1: Zone of inhibition values demonstrated by endophytic fungi from *Vitex rotundifolia* against test bacteria in plug assay

Endophytic fungi	Test bacteria (zone of inhibition in mm)						
	SA	BC	BS	ML	EF	EC	PA
Cochliobolus eragrostidis	-	14.00±0.00	14.83±0.29	-	-	-	-
Cochliobolus eragrostidis	-	-	-	-	-	-	-
Curvularia sp.	16.17±0.29	14.83±0.29	13.00±0.00	-	-	-	-
Curvularia sp.	-	-	-	-	-	-	-
Fungal sp. ARIZ B233	-	-	-	-	-	-	-
Guignardia mangiferae	-	12.33±0.58	14.00±0.00	-	-	-	-
Letendraea helminthicola	-	-	-	-	-	-	-
Nemania primolutea	-	-	-	-	-	-	-
Nemania primolutea	-	-	-	-	-	-	-
Paecilomyces sp.	16.00±0.00	16.17±0.29	15.00±0.00	-	-	-	-
Phoma sp.	-	-	-	-	-	-	-
Phoma sp.	-	8.00±0.00	-	-	-	-	-
Phoma sp.	-	-	-	-	-	-	-

Mean ± standard deviation, n=3; "-" = no activity against all test bacteria
SA: *Staphylococcus aureus*; BC: *Bacillus cereus*; BS: *Bacillus subtilis*; EF: *Enterococcus faecalis*; ML: *Micrococcus luteus*; EC: *Escherichia coli*; PA: *Pseudomonas aeruginosa*

Table 2: Zone of inhibition values demonstrated by endophytic fungi from *Ipomoea pes-caprae* against test bacteria in plug assay

Endophytic fungi	Test bacteria (zone of inhibition in mm)						
	SA	BC	BS	ML	EF	EC	PA
Bipolaris sp.	-	-	10.17±0.29	25.00±0.00	-	-	-
Bipolaris sp.	21.00±0.00	14.83±0.29	10.00±0.00	18.67±0.29	15.67±0.58	8.00±0.00	-
Bipolaris sp.	-	-	-	-	-	-	-
Bipolaris sp.	-	-	-	-	-	-	-
Cladosporium cladosporioides	-	-	-	-	-	-	-
Cochliobolus lunatus	-	-	-	-	-	-	-
Colletotrichum hippeastri	-	-	-	-	-	-	-
Colletotrichum sp.	-	-	-	-	-	-	-
Dothideomycete sp.	10.17±0.29	11.17±0.29	-	10.00±0.00	-	-	8.00±0.00
Dothideomycete sp.	-	-	-	-	-	-	-
Fungal endophyte sp g88	-	-	-	-	-	-	-
Fusarium equiseti	27.33±0.58	24.00±0.00	22.00±0.00	26.00±0.00	-	-	-
Guignardia mangiferae	-	-	-	-	-	-	-
Minimidochium sp.	8.33±0.47	8.33±0.24	13.33±0.58	10.83±0.29	-	8.67±0.24	-
Montagnulaceae sp.	24.67±0.29	27.33±0.58	19.83±0.29	24.00±0.00	-	-	-
Myrothecium sp.	8.33±0.47	8.33±0.47	8.33 ±0.24	-	-	-	-
Penicillium sp.	8.00±0.00	8.00±0.00	-	-	-	8.67±0.24	-
Phoma sp.	-	-	-	8.00±0.00	-	-	-
Phoma sp.	10.83±0.29	-	-	10.17±0.29	-	-	-
Pleosporales sp.	-	-	-	-	-	-	-
Xylaria sp.	-	-	-	-	-	-	-

Mean ± standard deviation, n=3; "-" = no activity against all test bacteria

SA: *Staphylococcus aureus*; BC: *Bacillus cereus*; BS: *Bacillus subtilis*; EF: *Enterococcus faecalis*; ML: *Micrococcus luteus*; EC: *Escherichia coli*; PA: *Pseudomonas aeruginosa*

Table 3: Zone of inhibition values demonstrated by manglicolous fungi against test bacteria in plug assay

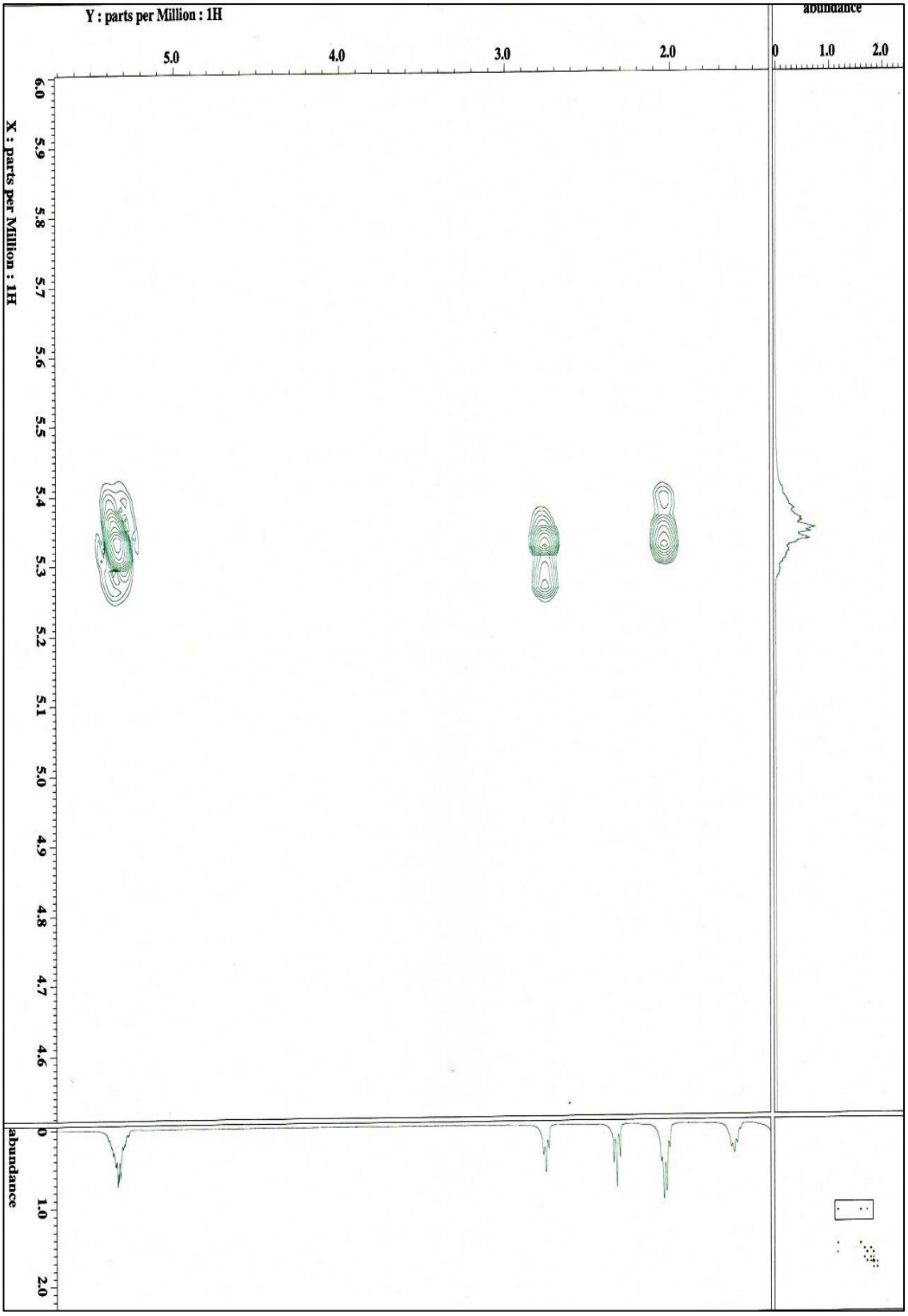
Manglicolous fungi	Test bacteria (zone of inhibition in mm)						
	SA	BC	BS	ML	EF	EC	PA
Corollospora maritima	-	10.33±0.236	10.167±0.24	-	-	-	-
Dactylospora haliotrepha (ISB001)	10.83±0.24	-	10.33±0.24	-	-	-	-
Dactylospora haliotrepha (ISB002)	11.00±0.41	-	10.83±0.24	-	-	-	-
Dactylospora haliotrepha (ISB003)	13.00±0.41	13.83±0.236	14.33±0.24	10.83±0.24	-	-	-
Dactylospora haliotrepha (ISB004)	13.00±0.41	14.33±0.236	13.83±0.24	-	-	-	-
Fusarium sp.	-	-	-	-	-	-	-
Henningsomyces sp.	14.67±0.24	14.33±0.236	13.67±0.24	10.33±0.24	-	-	-
MF 28	14.83±0.24	15.00±0.408	14.67±0.24	-	-	8.00±0.00	-
Saccardoella rhizophorae	15.83±0.24	16.17±0.236	15.00±0.41	14.67±0.24	-	8.00±0.00	-
Verruculina enalia (ISB005)	11.00±0.41	13.83±0.236	12.17±0.24	-	-	-	-
Verruculina enalia (ISB006)	10.83±0.24	12.17±0.236	11.00±0.41	-	-	-	-

Mean ± standard deviation, n=3; "-" = no activity
SA: *Staphylococcus aureus*; BC: *Bacillus cereus*; BS: *Bacillus subtilis*; EF: *Enterococcus faecalis*; ML: *Micrococcus luteus*; EC: *Escherichia coli*; PA: *Pseudomonas aeruginosa*

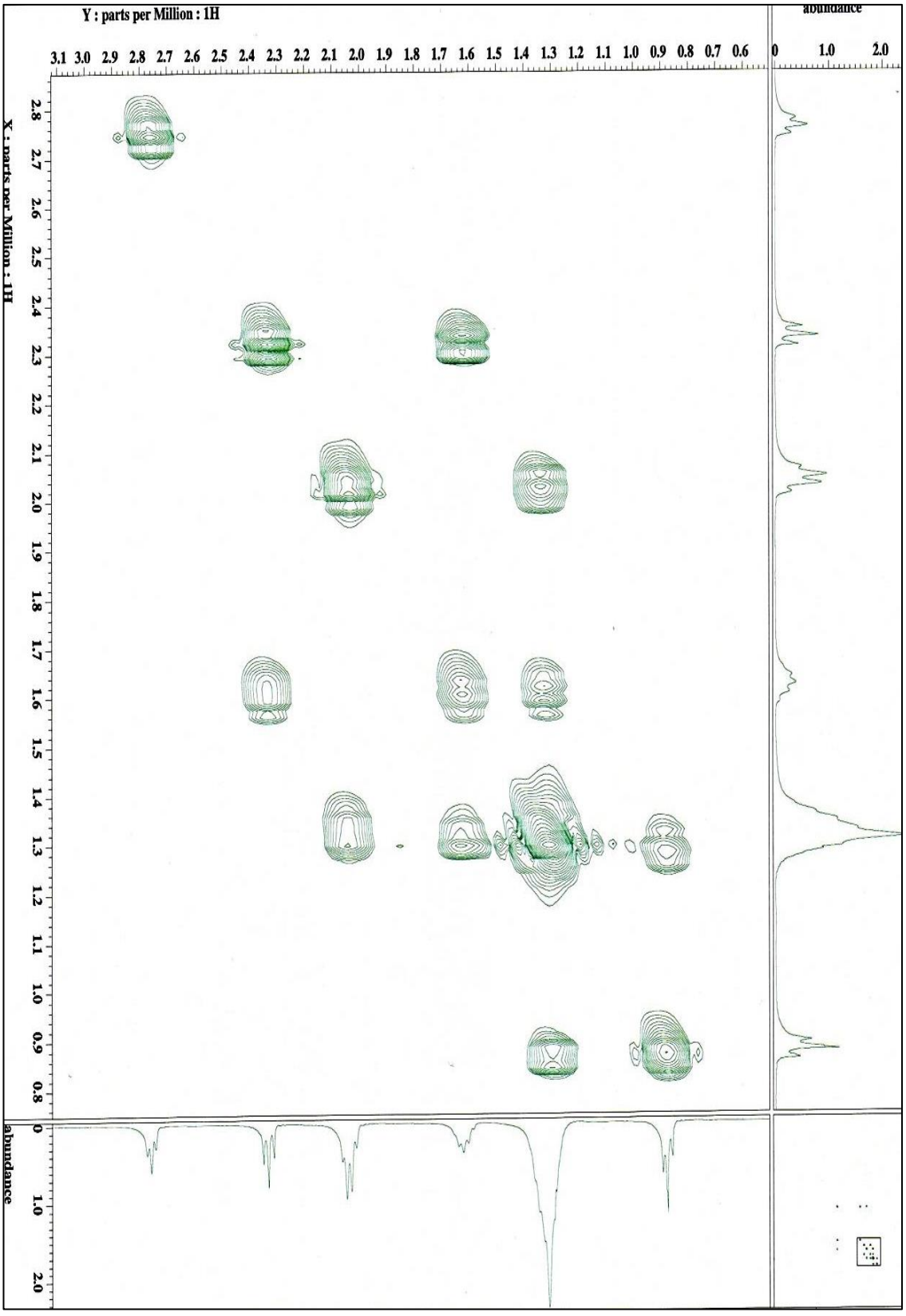
APPENDIX 4

2-Dimensional NMR spectrum of linoleic acid in $CDCl_3$

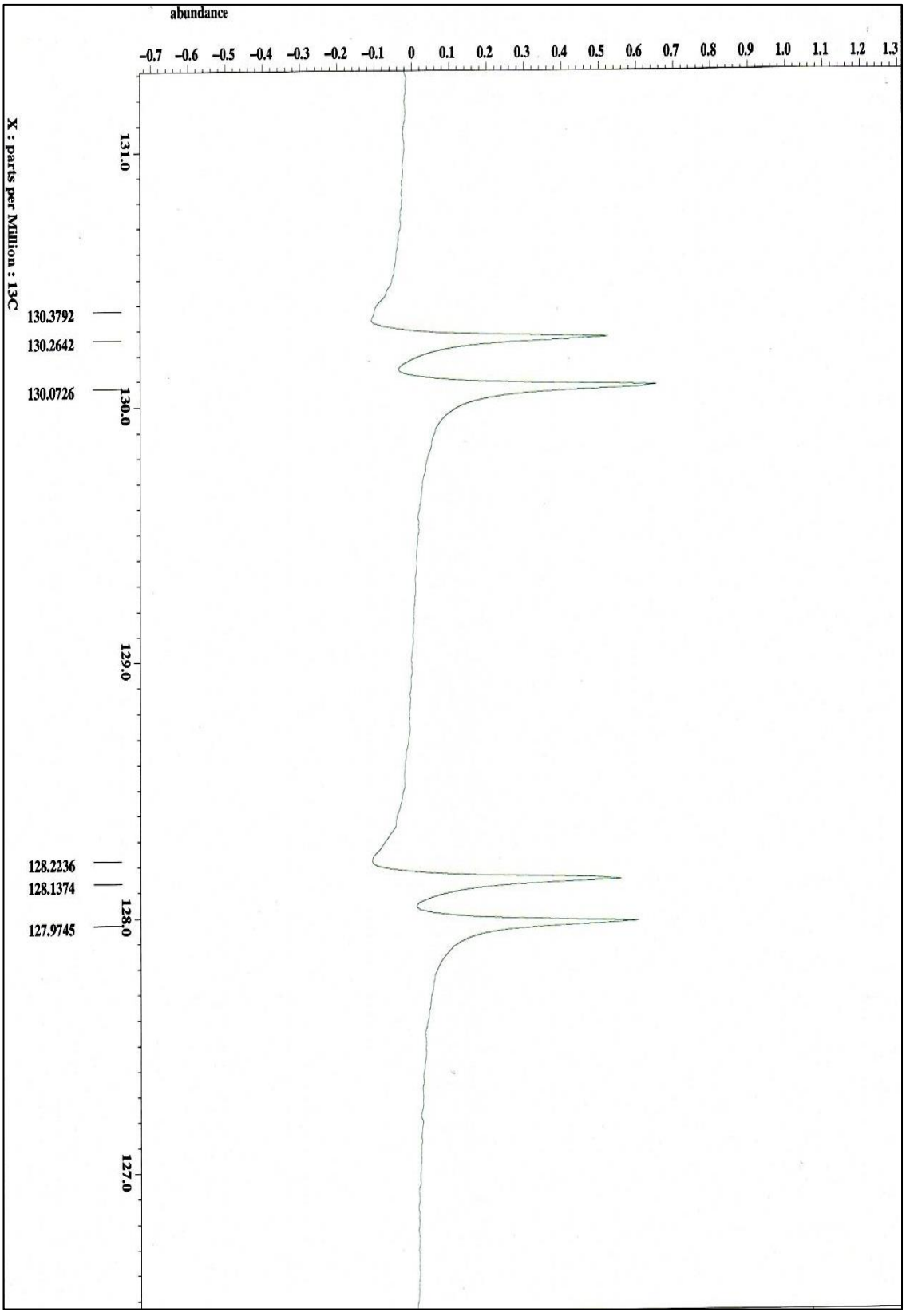
Correlation spectroscopy (COSY) spectrum



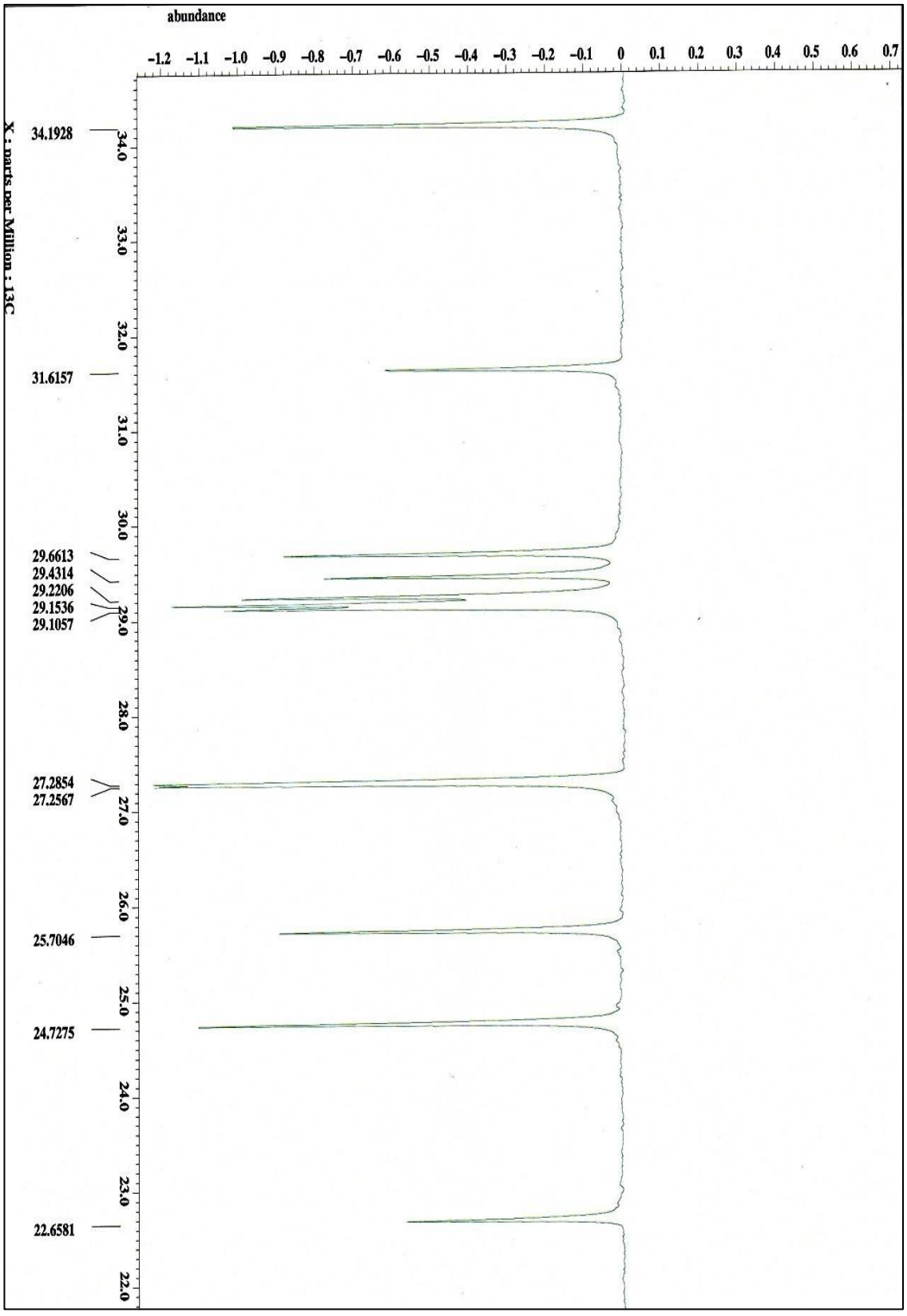
COSY spectrum (continued)



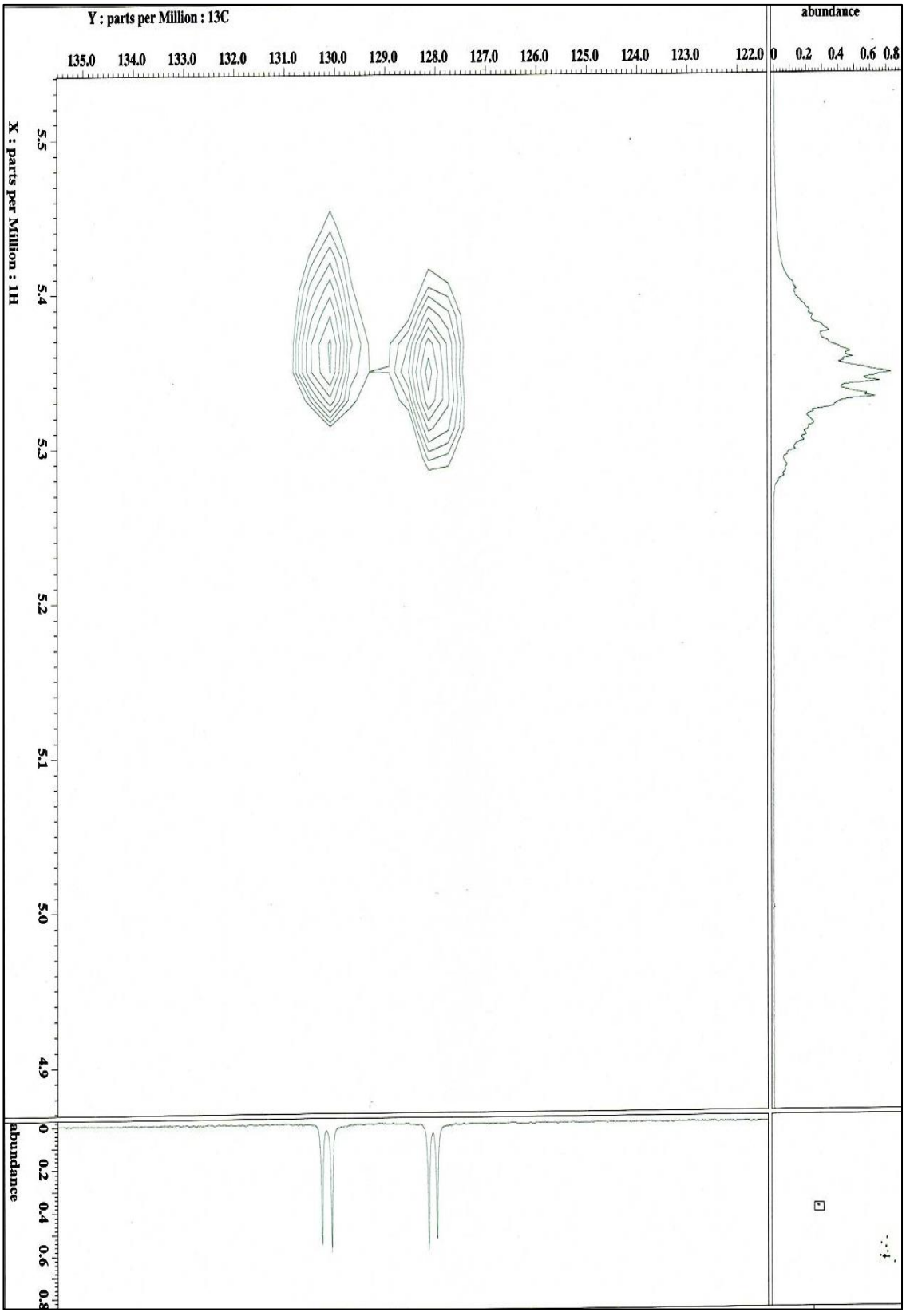
Distortionless enhancement by polarization transfer (DEPT) spectrum



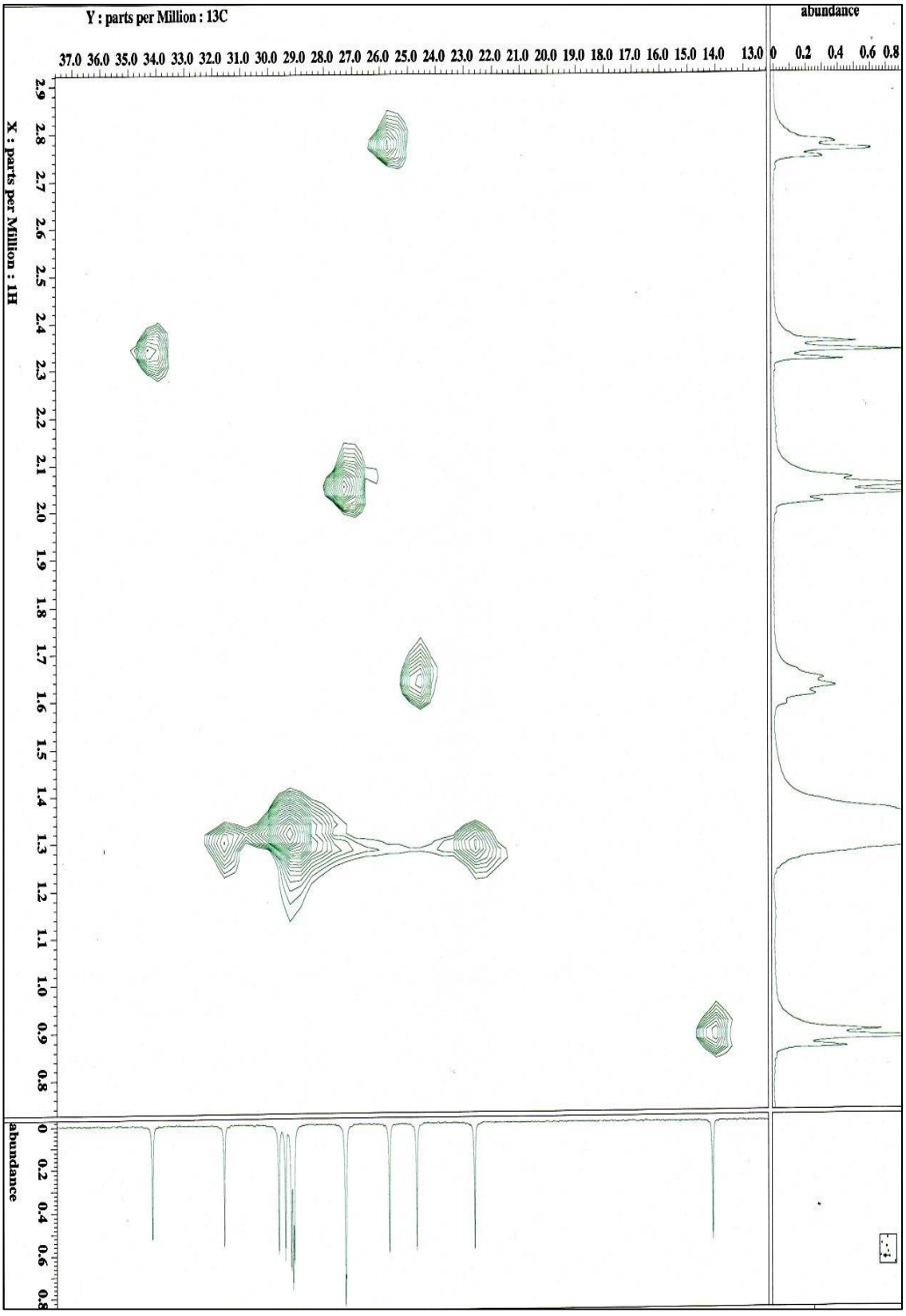
DEPT spectrum (continued)



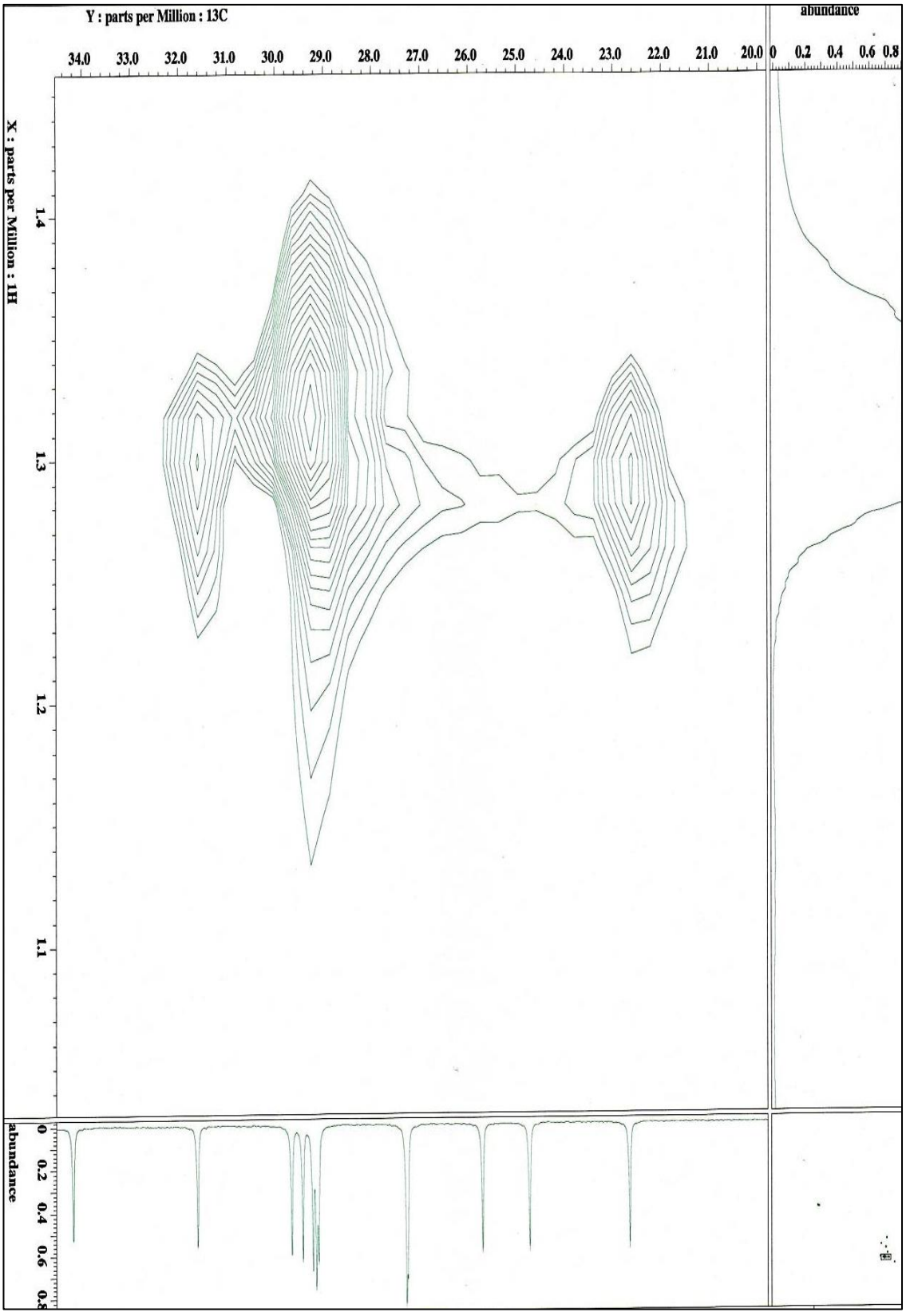
Heteronuclear single-quantum correlation spectroscopy (HSQC) spectrum



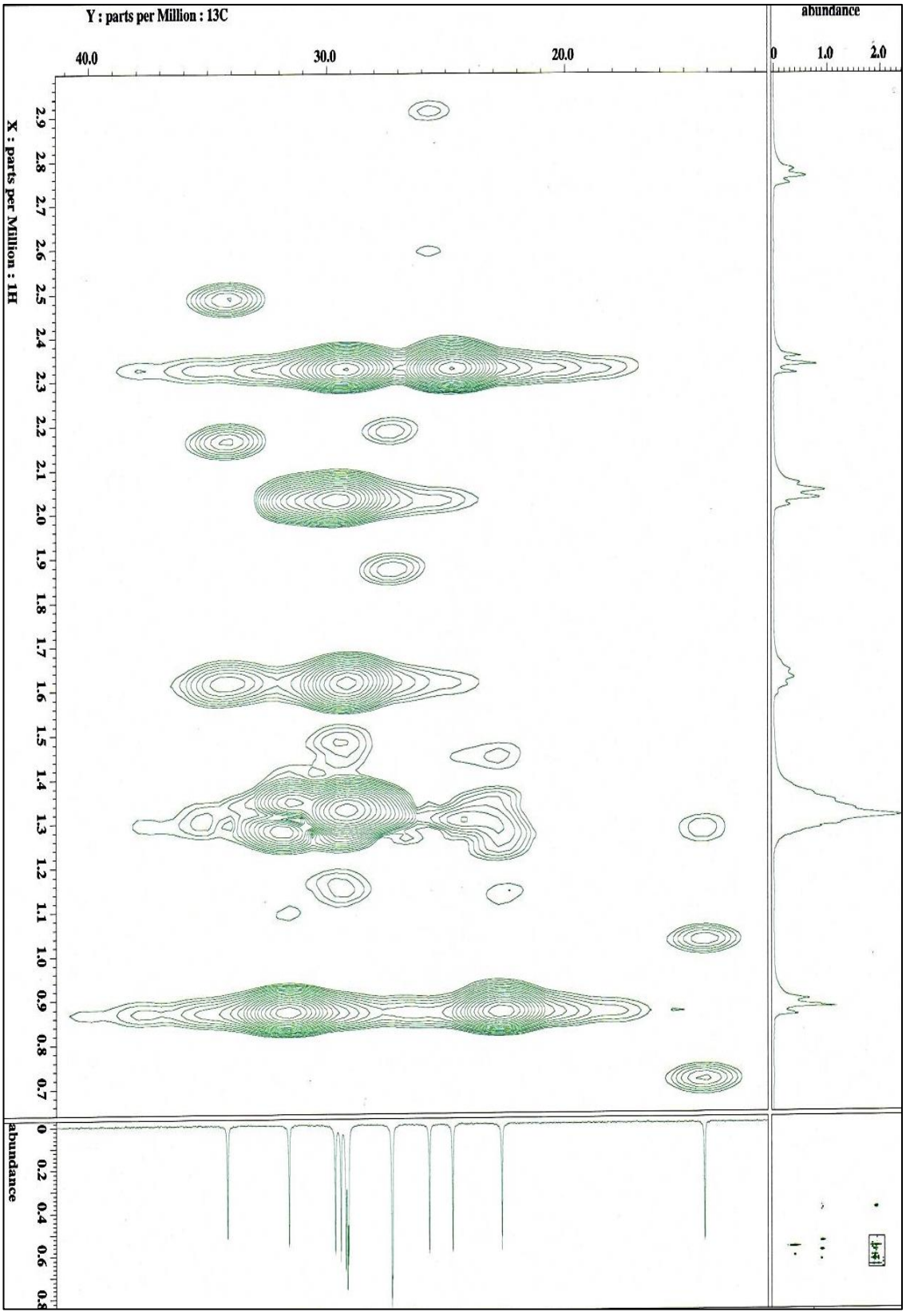
HSQC spectrum (continued)



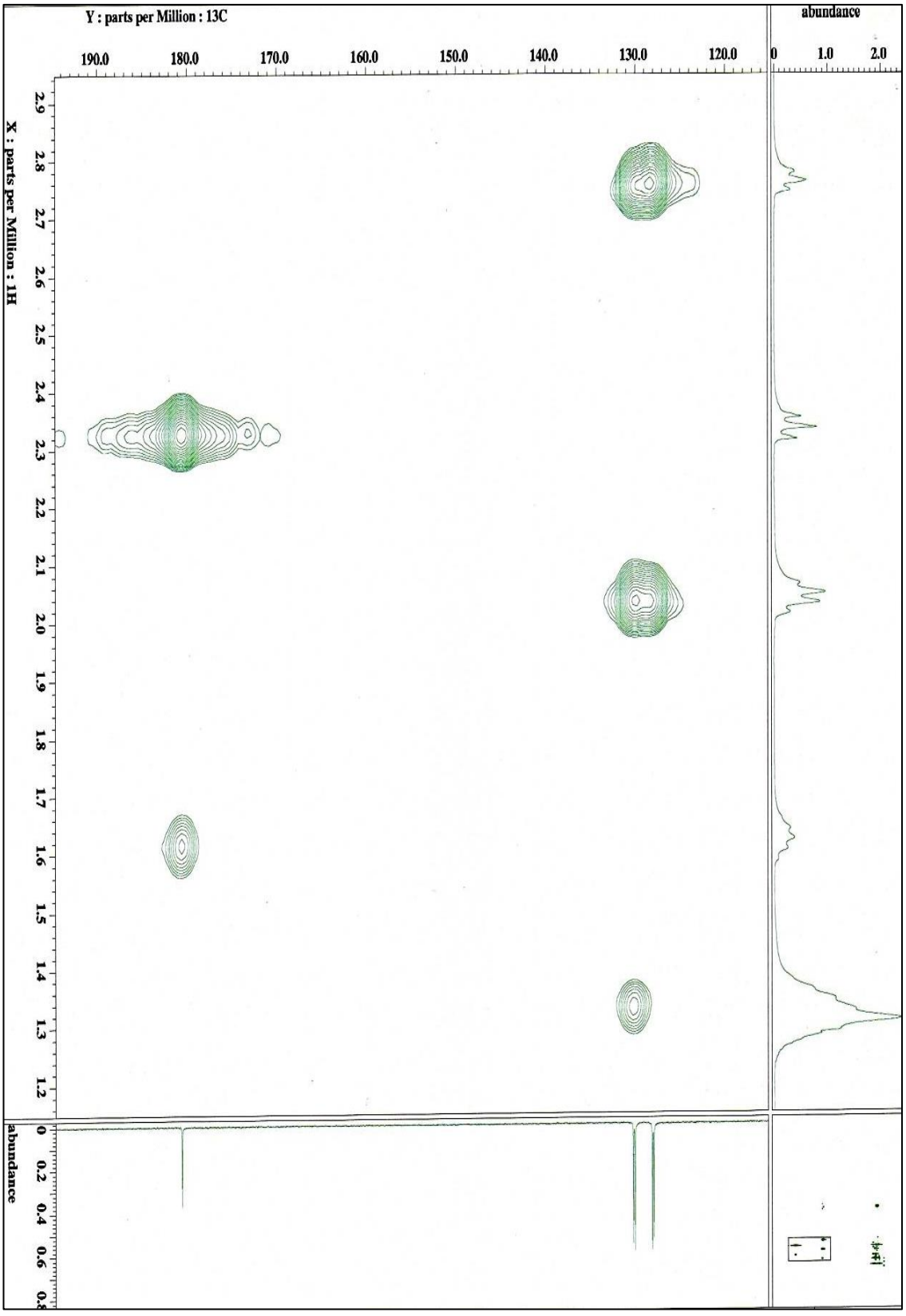
HSQC spectrum (continued)



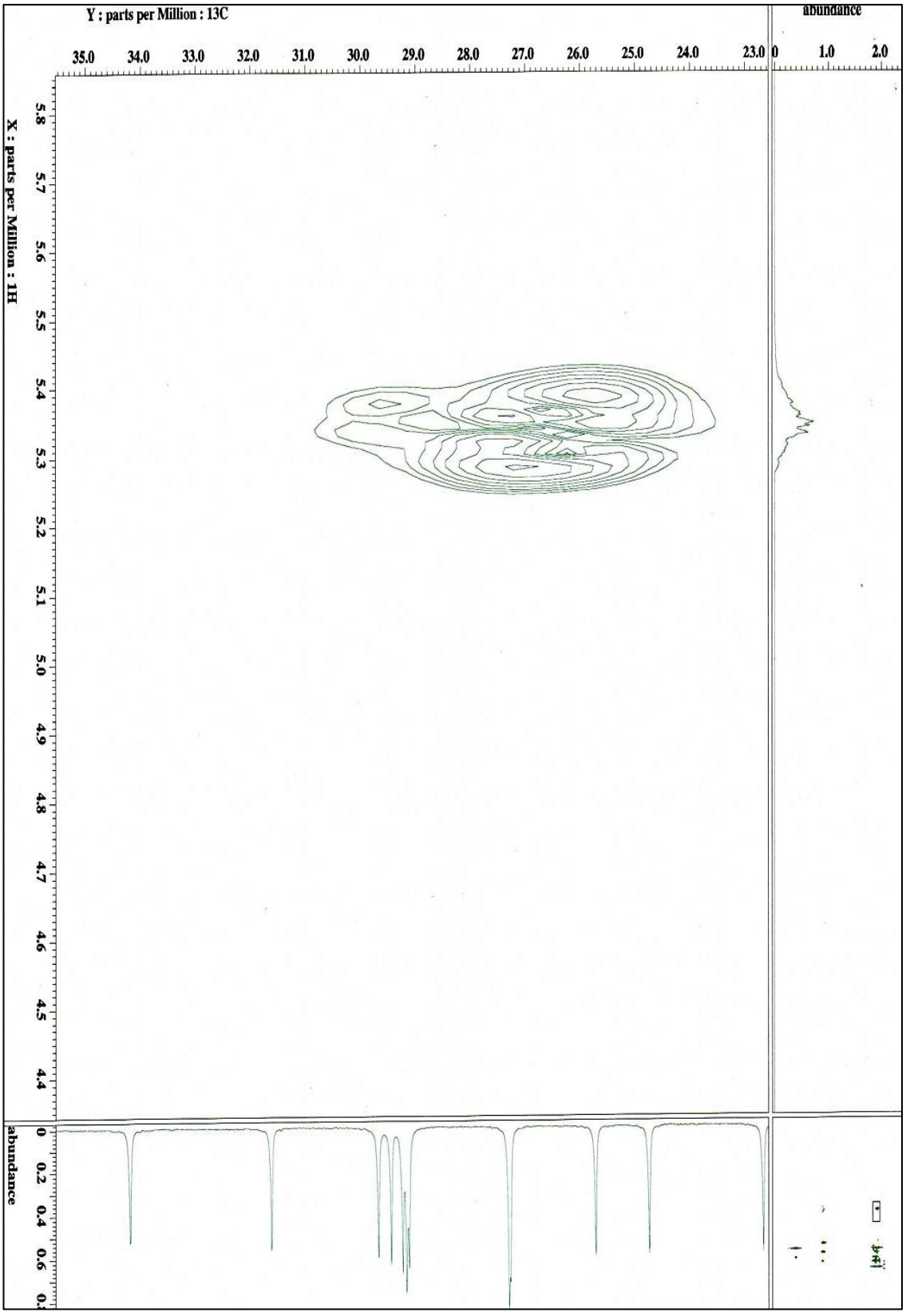
Heteronuclear multiple-bond correlation spectroscopy (HMBC) spectrum



HMBC spectrum (continued)



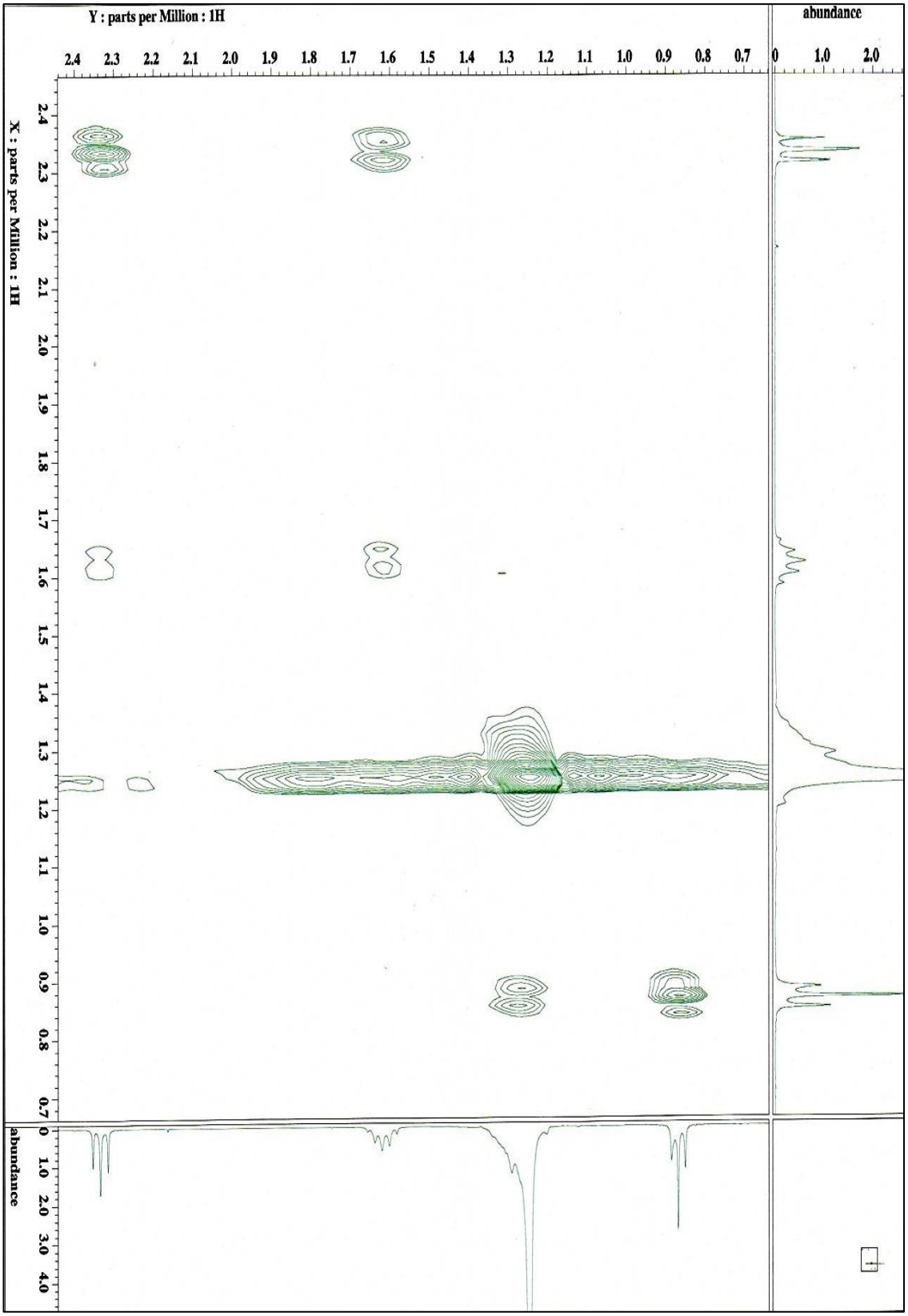
HMBC spectrum (continued)



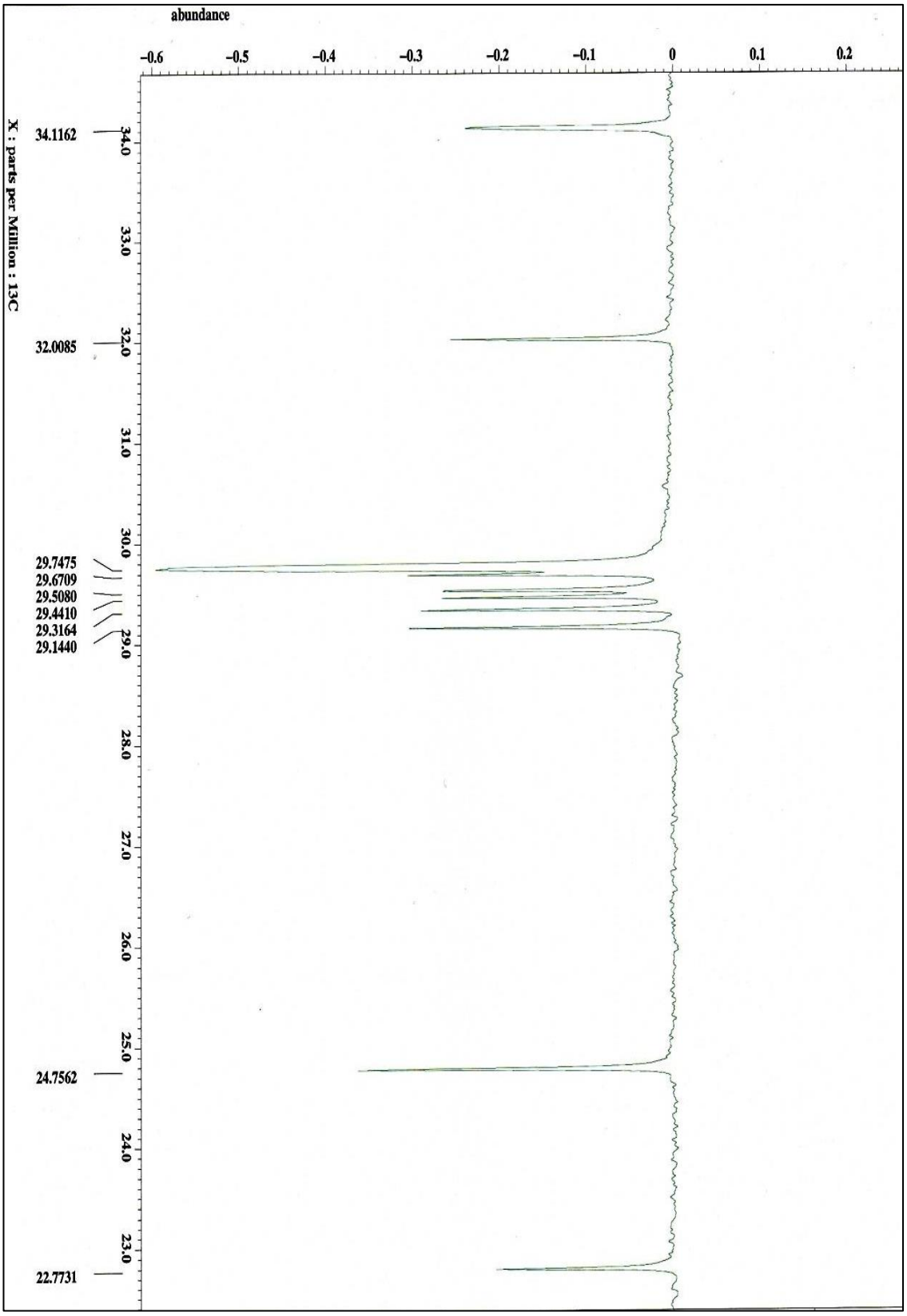
APPENDIX 5

2-Dimensional NMR spectrum of palmitic acid in $CDCl_3$

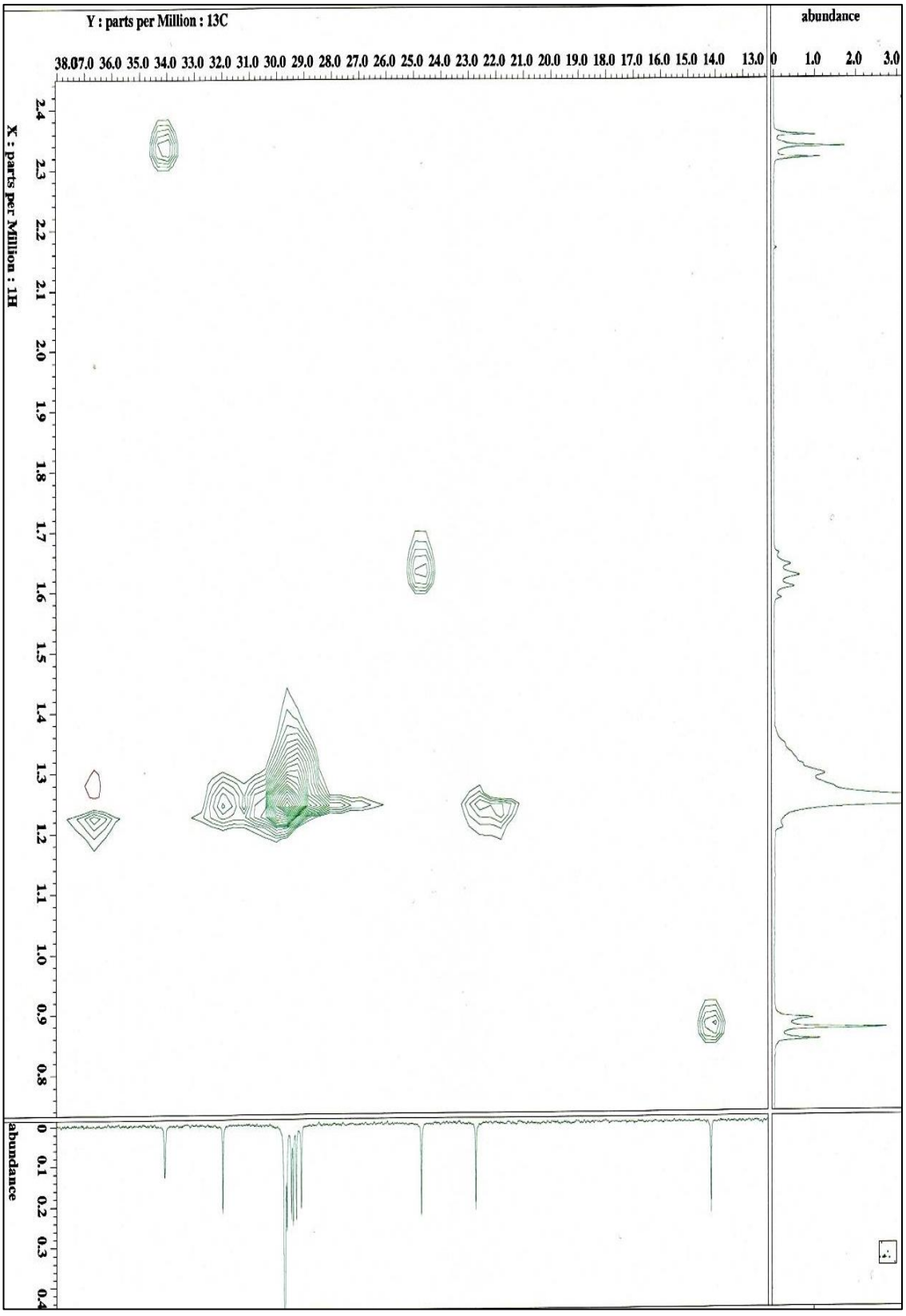
COSY spectrum



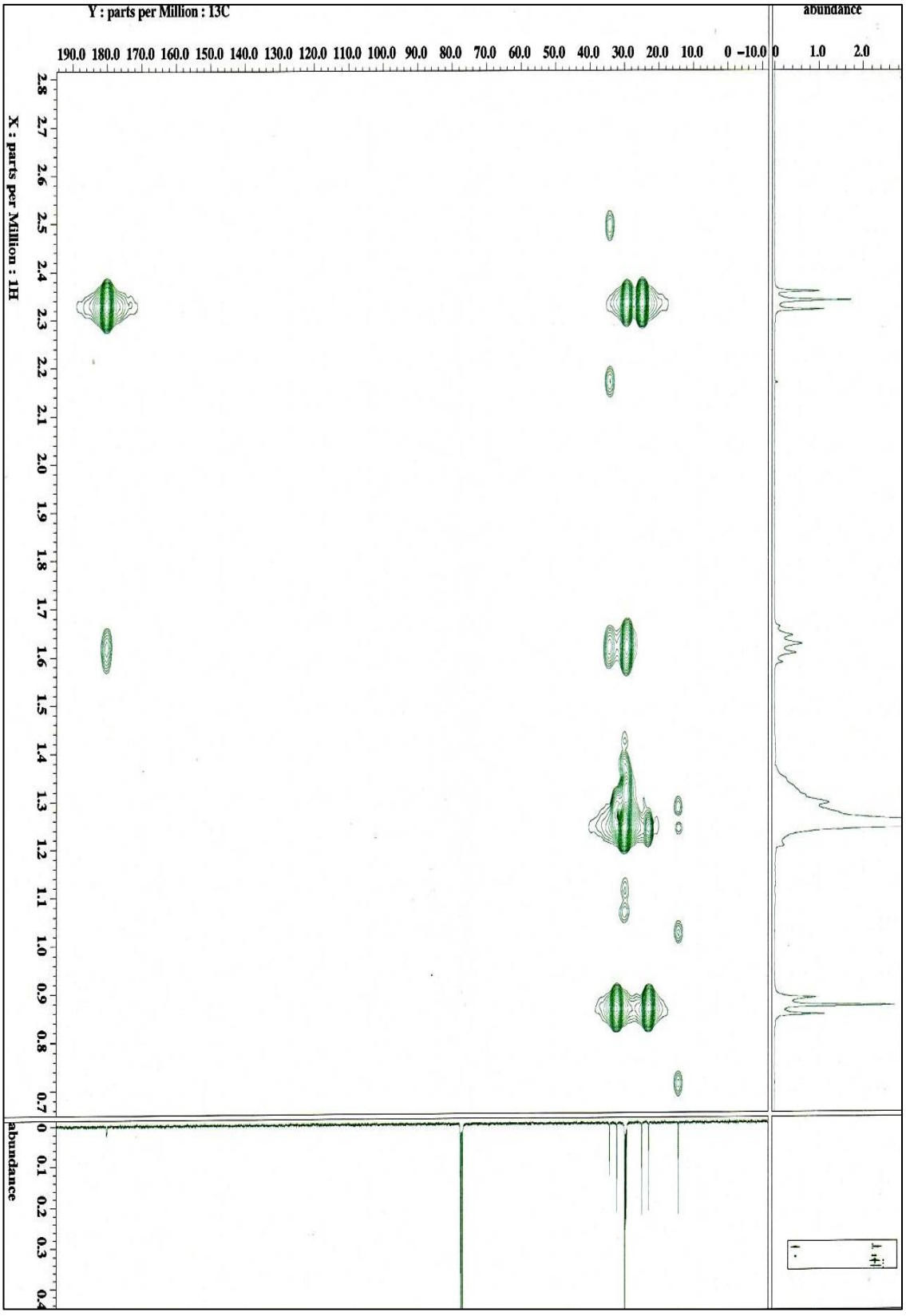
DEPT spectrum



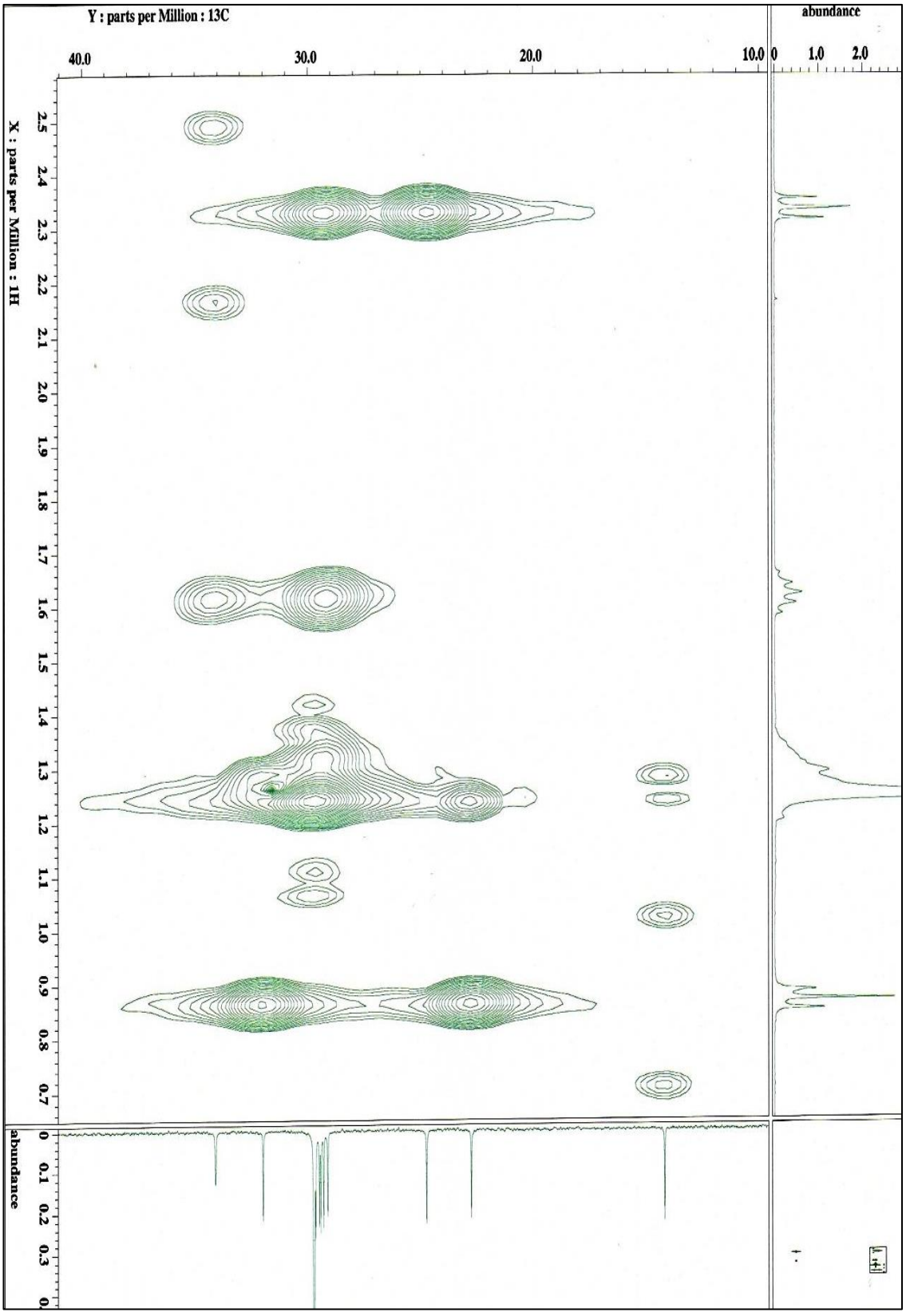
HSQC spectrum



HMBC spectrum



HMBC spectrum (continued)



APPENDIX 6

List of presentation abstracts

Paliany, A.S., Alias, S.A., Awang, K. (2012). Assessing the antibacterial bioactivity of saprophytic and endophytic marine fungi from the east and west coastline, Peninsular Malaysia. Abstract in book, 17th Biological Sciences Graduate Congress. Chulalongkorn University, Thailand.

BSGC 2012- Theme 2: Applied Science and Biotechnology
Presentation type: Poster presentation

Assessing the antibacterial bioactivity of saprophytic and endophytic marine fungi from the east and west coastline, Peninsular Malaysia

Audra Shaleena Paliany[1], Siti Aisyah Alias[1,2*], Khalijah Awang[3]
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Majority of drugs today are derived from plants and microorganism. The harsh environment of the marine ecosystem offers promising potential for new bioactive compounds. A total of 11 saprophytic and 46 endophytic fungi were evaluated for their antibacterial activity. The saprophytic fungi were isolated from various manglicolous materials in Malaysia while the endophytic fungi were isolated from two marine associated plants, *Ipomoea pes-caprae* and *Vitex rotundifolia*. These two plants were found along the shoreline from west and east coastal regions in Malaysia. The minimum inhibitory concentration (MIC) of all saprophytes and preliminary screening of all 46 endophytes collected were evaluated against 7 bacterial strains consisting of 2 Gram negative and 5 Gram positive bacteria. Results for MIC displayed potent antibacterial activity of 4 saprophytic fungi strains; *Dactylospora haliotrepha* (tioman), *Sacardoella rhizophorae*, *Henningsomyces* sp. and an unidentified species, MF 28 against 4 Gram positive bacteria namely *Staphylococcus aureus*, *Bacillus cereus*, *Micrococcus luteus* and *Bacillus subtilis*. The endophytic fungi were screened using the plug assay method and the bacterial susceptibility index was evaluated for all 7 bacteria. Results obtained showed that *Bacillus cereus* had the highest susceptibility index at 0.3696% followed by *Staphylococcus aureus* at 0.3043%, *Bacillus subtilis* at 0.2609% and *Micrococcus luteus* at 0.2391%. The lowest bacterial susceptibility index was recorded by *Escherichia coli* at 0.0217%. Further work will be focussing on the species identification of all isolated endophytes through molecular sequencing of their ITS sequences and the chemical characterization and elucidation of pure compounds from the active fungi strains.

Paliany, A.S., Sivasothy, Y., Alias, S.A., Awang, K. (2013). The antibacterial activity of marine derived fungi from Peninsular Malaysia. Abstract in book, International Marine and Freshwater Mycology Symposium. China National Convention Center, China.

IMFMS 2013- Session 1: Bioactive Compounds of Marine Origin
Presentation type: Oral presentation

The antibacterial activity of marine derived fungi from Peninsular Malaysia

Audra Shaleena Paliany[1], Yasodha Sivasothy[2], Khalijah Awang[2], Siti Aisah Alias[1, 3*]
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[2] *Department of Chemistry, University of Malaya, 50603, Kuala Lumpur, Malaysia*
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Over the past 2 decades, scientist have come to acknowledge the growing importance of marine and marine-related resources such as plants, sponges, algae and microbes, for new drugs, pesticides and anti-fouling substances. Marine fungi have been recognized as a rich of novel bioactive metabolites with potential biomedical use. In our study, we explored the antibacterial activity of marine derived endophytic and saprophytic fungi. The endophytic fungi in this study was isolated from 2 marine associated plants; *Vitex rotundifolia* L. f. and *Ipomoea pes-caprae* (L.) R.Br., from the coastlines of Peninsular Malaysia. The saprophytic fungi in this study were from the mangrove habitat. A total of 12 and 21 endophytic fungi were isolated from *Vitex rotundifolia* and *Ipomoea pes-caprae* respectively while 10 saprophytic fungi were isolated from the mangrove environment. The plug assay and broth microdilution assay were adopted as antibacterial screening methods of the fungi strains. Results showed that the endophytes from *Ipomoea pes-caprae* exhibited a broader spectrum of antibacterial activity covering both Gram positive and negative bacteria in comparison to the endophytes from *Vitex rotundifolia*. The saprophytic fungi, *Saccardoella* sp. showed a broad spectrum antibacterial activity against both Gram positive and negative bacteria. The chemical analyses of the secondary metabolites from *Saccardoella* sp. revealed 2 chemical compounds with antibacterial activity. The search for novel metabolites of fungal origin in Malaysia is still at its preliminary stages with very few reports published. Thus this study may prove crucial to pave the way for further discovery of novel fungal metabolites with pharmacological values.

Paliany, A.S., Sivasothy, Y., Awang, K., Idid, M.R., Alias, S.A. (2014). Antibacterial Potential of Marine Derived Fungi from Peninsular Malaysia and Metabolites of *Saccardoella rhizophorae*. Abstract in book, International Conference on Fungal Biodiversity and Biotechnology. Mae Fah Luang University, Thailand.

Presentation type: Poster presentation

Antibacterial Potential of Marine Derived Fungi from Peninsular Malaysia and Metabolites of *Saccardoella rhizophorae*

Audra Shaleena Paliany[1], Yasodha Sivasothy[2], Khalijah Awang[2], Mohammed Rizman Idid[3] Siti Aisyah Alias[1,3*]
[1]*Institute of Biological Sciences, University of Malaya, 50603, Kuala Lumpur, Malaysia*
[2]*Department of Chemistry, University of Malaya, 50603, Kuala Lumpur, Malaysia*
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Abstract:
The past 2 decades has seen the discovery of numerous new and novel fungal metabolites of promising pharmacological significance. Marine fungi in particular, have now been recognized as good harbors of important novel bioactive metabolites with potential biomedical applications. In an effort to tap into natural products harboured by marine derived fungi in Malaysia, selected marine derived endophytic and manglicolous fungi from the coastlines of Peninsular Malaysia were investigated for their antibacterial potential. A total of 12 and 21 endophytic fungi were isolated from the marine associated plants, *Vitex rotundifolia* and *Ipomoea pes-caprae* respectively while 10 manglicolous fungi were isolated from various decaying mangrove wood of Peninsular Malaysia. In preliminary experiments, the plug assay was employed to study the antibacterial activities of all 33 fungi isolates. Fifteen or 45.45% of the endophytic fungi isolates and 9 or 90% of the manglicolous fungi isolated displayed antibacterial activities against at least one of the test bacteria. The endophytic fungi from *I. pes-caprae* displayed higher antibacterial potential in comparison to the endophytic fungi from *V. rotundifolia* especially the strains, *Minimidochium* sp. and *Bipolaris* sp. which displayed antibacterial activities against 5 or more test bacteria. The fungi isolates that exhibited good antibacterial activities in the plug assay was further analysed through the broth microdilution assay where the fungi strains *Minimidochium* sp and *Saccardoella rhizophorae* exhibited promising antibacterial activities with minimum inhibitory concentrations not higher than 0.5mg/ml. Bioactivity-guided fractionation of *S. rhizophorae* extract resulted in the isolation of high concentrations of palmitic and linoleic acid thereby suggesting *Saccardoella rhizophorae* to be a promising biodiesel source. The search for novel metabolites of fungal origin in Malaysia is still at its preliminary stages with very few reports published thereby this study may prove crucial to pave the way for further discovery of fungal metabolites with commercial values.

APPENDIX 7

List of abstracts for journal publication

(Accepted)

Marine Derived Fungi of Peninsular Malaysia-a Biochemical Perspective

Audra Shaleena Paliany[a], Yasodha Sivasothy[b], Khalijah Awang[b], Mohammed Rizman-Idid[a,c] and Siti Aisyah Alias*[a,c]

[a]Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia,

[b]Department of Chemistry, University of Malaya, Kuala Lumpur 50603, Malaysia

[c]Institute of Ocean and Earth Sciences, University of Malaya, Kuala Lumpur 50603, Malaysia

Abstract

In an effort to tap into natural products harboured by marine derived fungi in Malaysia, selected marine derived endophytic and manglicolous fungi from the coastlines of Peninsular Malaysia were investigated for their antibacterial potential. Forty-one strains were isolated from marine associated plants, comprised and comprised 11 and 18 endophytic strains from *Vitex rotundifolia* and *Ipomoea pes-caprae* respectively, while 10 manglicolous strains were from decaying mangrove wood collected in Peninsular Malaysia. In preliminary experiments, a plug assay was employed to study the antibacterial activities of all 39 fungi isolates. Fifteen of the endophytic isolates and nine of the manglicolous isolates displayed antibacterial activities against at least one of the test bacteria. Based on the plug assay, the endophytic fungi from *I*pomoea *pes-caprae* were shown to display higher antibacterial potential in comparison to the endophytic fungi from *V*itex *rotundifolia*. In particular, *Minimidochium* sp. and *Bipolaris* sp. (ISB0014) displayed antibacterial activities against 5 or more test bacteria. Potential fungi isolates with good antibacterial activities were further analysed through a broth microdilution assay. *Minimidochium* sp. and *Dyfrolomyces rhizophorae* exhibited promising antibacterial activities with minimum inhibitory concentrations not higher than 0.5 mg/ml. Bioactivity-guided fractionation of *D. rhizophorae* extracts resulted in the isolation of fatty acids, palmitic and linoleic acid. Though ubiquitous in nature, linoleic acid is known as an essential health supplement and both fatty acids are used as biodiesel replacing conventional fuels.

Keywords: Natural products, Antibiotics, Manglicolous fungi, Endophytic fungi, Bioactivity.

(Published)

Pahangensin A and B, two new antibacterial diterpenes from the rhizomes of *Alpinia pahangensis* Ridley

Yasodha Sivasothy[a], Halijah Ibrahim[b], Audra Shaleena Paliany[b], Siti Aisyah Alias[b], Khalijah Awang[a*]

[a] *Department of Chemistry, Faculty of Science, University Malaya, 50603, Kuala Lumpur, Malaysia*

[b] *Institute of Biological Sciences, Faculty of Science, University Malaya, 50603, Kuala Lumpur, Malaysia*

Abstract

The rhizomes of *Alpinia pahangensis* Ridley yielded a new bis-labdanic diterpene for which the name pahangensin A (**1**) was proposed along with a new labdane diterpene, pahangensin B (**2**). Their structures were elucidated by spectroscopic methods including, 1D and 2D NMR techniques and LCMS-IT-TOF analysis. Pahangensin A (**1**) was found to be an antibacterial agent against *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis* with MIC values less than 100 µg/mL, respectively. Pahangensin B (**2**) exhibited antibacterial activity (MIC < 100 µg/mL) against *Bacillus cereus*.

Keywords: *Alpinia pahangensis* Ridley, Zingiberaceae, bis-labdanic diterpene, labdane diterpene, pahangensin A, pahangensin B, antibacterial agent

(Published)

A new bis-labdanic diterpene from the rhizomes of *Alpinia pahangensis*

Yasodha Sivasothy[1], Halijah Ibrahim[2], Audra Shaleena Paliany[2], Siti Aisyah Alias[2], Nurur Raudzah Md. Nor[3], Khalijah Awang[1]*

[1] *Department of Chemistry, Faculty of Science, University Malaya, 50603, Kuala Lumpur, Malaysia.*

[2] *Institute of Biological Sciences, Faculty of Science, University Malaya, 50603, Kuala Lumpur, Malaysia.*

[3] *Chemistry Department, Center of Foundation Studies, International Islamic University Malaysia, Petaling Jaya, 46350, Selangor, Malaysia.*

Abstract

The rhizomes of *Alpinia pahangensis* Ridley yielded a new bis-labdanic diterpene for which the name pahangensin C (**1**) was proposed along with twelve known analogues (**2**-**13**). The structure of **1** was elucidated via spectroscopic methods including, 1D and 2D NMR techniques and LCMS-IT-TOF analysis. Compounds **2** and **12** were isolated for the first time from the genus *Alpinia*. This is the second occurrence of compounds **2** and **12** in the Zingiberaceae. Selected analogues exhibited moderate to strong inhibitory activity against *Staphylococcus aureus* and *Bacillus cereus*.

Keywords: *Alpinia pahangensis* Ridley, Zingiberaceae, bis-labdanic diterpene, labdane diterpene, pahangensin C